VARIATION IN ECOTYPES OF POA PRATENSIS[1]

D. C. SMITH,[2] E. L. NIELSEN,[3] AND H. L. AHLGREN[4]

Introduction

The existence of apomictic seed development appears to be a fact well established for *Poa pratensis* L. Though apomixis is the usual form of reproduction, many plants may produce seed frequently following amphimixis. The species is highly polymorphic, and extensive investigations have been made to determine the extent and nature of the variability. The possibilities of selection of superior plants and of their subsequent propagation as improved strains have been recognized. Commercial seed of Kentucky bluegrass is heterogeneous, and progenies from it may give a wide complex of plant types. The method of harvesting commercial seed and the possible selection for seed production characters rather than for vegetative vigor may be accompanied by a diminished pasture value. In most areas of the Northeastern and North Central states, however, the species is now sufficiently prevalent to be considered as naturalized, and it is seldom seeded for pasture purposes.

Breeding investigations with Kentucky bluegrass were begun at the Wisconsin Experiment Station in 1936. The principal objectives have been (*a*) the isolation and testing of superior biotypes for pasture and turf purposes, (*b*) the testing of the breeding behavior of such biotypes, and (*c*) the determination of the nature of variation in the species.

The present paper presents the results of studies of variation in several characters. A large number of seeded panicles for use in progeny studies was collected in Wisconsin and other states where the grass is relatively common.

Review of literature

BRITTINGHAM (**4**) has comprehensively reviewed and discussed the earlier literature. Several workers have studied apomixis, but not all have presented results of progeny tests. ÅKERBERG (**1**) found that various selected families segregated from 5.9 to 13.4% sexual (aberrant) plants. Thirty-seven of forty-four native collections from Sweden, Germany, and Norway were "practically" constant morphologically, the remaining seven each showing great variation. ÅKERBERG (**2**) also studied families from collections made in Sweden, Norway, Finland, Denmark, Germany, and England. Five to ten plants of each family were grown. Seventy-nine families averaged 3.9% aberrant. Within each of nine families no two plants were alike, while each of five families was essentially homogeneous.

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[2, 4] Professor of Agronomy, Wisconsin Agricultural Experiment Station; [3] Associate Agronomist, Division of Forage Crops and Diseases, Bureau of Plant Industry, Soils and Agricultural Engineering, U.S. Department of Agriculture. The writers are indebted to the Statistical Laboratory of the Wisconsin Experiment Station for aid in the statistical calculations and to R. J. MUCKENHIRN for counsel regarding the soil classifications utilized.

TINNEY and AAMODT (**14**) investigated 102 progenies numbering 21 to 243 plants each—a total of 11,696 plants. The strains were of domestic and foreign origin as follows:

Wisconsin, 31	Sweden, 6
Virginia, 1	Denmark, 1
Missouri, 2	England, 1
Ottawa, Canada, 34	India, 1
Guelph, Canada, 21	Russia, 2
Norway, 2	

The average percentage of aberrants for all progenies was 1.6, with values ranging from 0 to 21.9%. Forty-eight progenies appeared to be uniform. The thirty-one Wisconsin collections averaged 1.65% aberrant, while the fourteen other nonuniform progenies averaged 3.65% aberrant.

BROWN (**6**) found that progenies of six lots of selected plants produced 0.09–18.18% aberrant types. Five others were apparently uniform. Fifty-five to 133 plants of each lot were grown.

BRITTINGHAM (**4**) observed an average of 14.8% aberrants in 115 progenies—approximately 10,000 individuals—resulting from open pollination. Four of the 115 progenies showed complete uniformity; one had the maximum of 90.5% aberrants. This material originated from permanent pastures, commercial seed lots, numbered experiment-station strains, and foreign-plant introductions.

EDGECOMBE (**8**) studied twenty-nine progenies of *P. pratensis;* all but one were considered to be uniform or nearly so. MUSGRAVE (**11**) concluded that about 90% of all types of *P. pratensis* observed reproduced seed apomictically.

BROWN (**5**) gave a brief summary of the taxonomic status of *P. pratensis*, indicating that, since 1811, eleven classifications by systematists had established from five to twenty-two subdivisions of the species. He considered *P. pratensis* to be composed of two primary groups as based upon general morphological features.

TINNEY and AAMODT (**14**) concluded that the intensity of grazing and other ecological factors, including locality of origin, showed little effect upon the selection of particular morphological types. WELLHAUSEN and WEIBEL (**15**) reported that plants from populations in "pastures which had been maintained in a good stage of productivity over a long period of years" were generally more productive and much more resistant to leaf rust and mildew than comparable plants from pastures on areas of low fertility, particularly in relation to calcium and phosphorus. Plants from both types of pastures were compared under the same conditions.

ÅKERBERG (**2**) concluded that generally the form complexes of Eu-*pratensis* did not seem to be confined to any particular locality. The form complexes *irrigata*, *angustifolia*, and *alpigena*, however, were thought to occur in somewhat different habitats. The Eu-*pratensis* forms were observed to be highly variable and widely scattered, the others, including *alpigena*, being less so. Differences were not entirely clear, however.

Classification of progeny plants as to agreement in type with the parent form has been based upon relative morphologic similarity at any period of the growing season. MYERS (**12**) has pointed out that classification upon this basis may be uncertain, since many apparently normal plants produce progenies of varying character and may, therefore, be considered to be aberrant. Similar results were reported by SMITH and NIELSEN (**13**). BRITTINGHAM (**4**) has suggested that increased mortality in sexual types should also be considered in relation to variation. However, when certain obvi-

ous limitations of the criteria used are recognized, the percentage of aberrant (sexual) plants in a progeny has been generally accepted as an accurate index of the extent of sexual reproduction.

BRITTINGHAM (4) noted no significant differences to exist in percentages of aberrant frequency when comparing collections from different sources. Variability was lost, this accounting for the lack of progenies from those states.

In making the collections no discrimination was shown as to location. Panicles were obtained from roadsides, fence rows, undergrazed and overgrazed pastures, and waste land. Some selection may have occurred, since only relatively well-developed panicles containing aver-

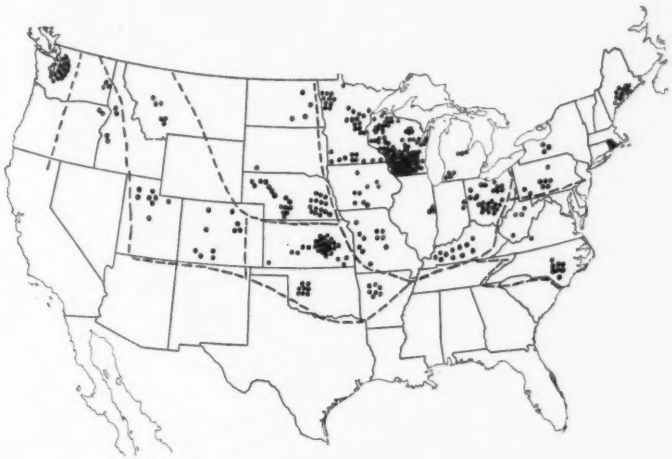


FIG. 1.—Distribution of 1941 collections of *Poa pratensis* in the United States. Dotted lines indicate general regional boundaries.

was somewhat lower, however, in many progenies of plants grown from commercial seed.

Material and methods

Progenies from a total of 800 collections from twenty-seven states were studied in the seasons 1941–44; these included 541 from Wisconsin. The distribution of the collections in the United States for 1941 is shown in figure 1 and that for Wisconsin in figure 2. The writers are indebted to a number of local investigators for many of the collections from other states. Unfortunately, most of the material from Illinois and Indiana

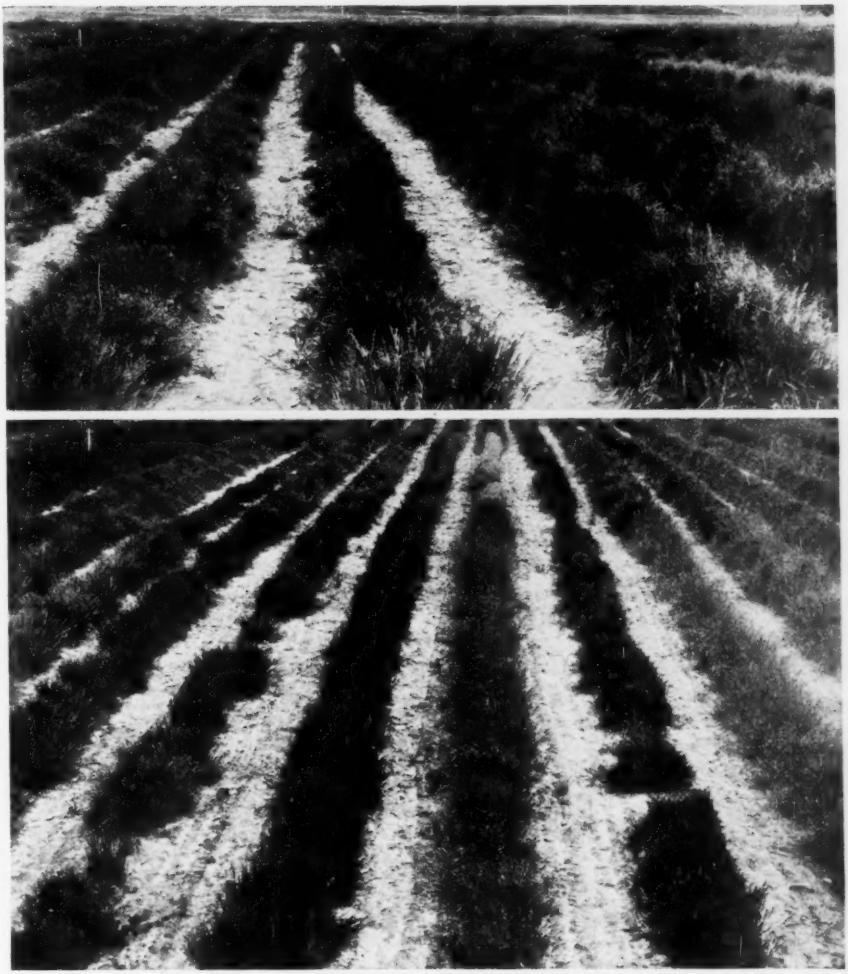
age or larger amounts of seeds were taken. Otherwise, relative culm height, texture, vigor, and other characters were ignored.

Progenies were grown from single panicles in a method similar to that described by TINNEY and AAMODT (14). Individual lots of seed were sown in steamed soil in small bread pans. Exceptions to the procedure were necessary when commercial lots, bulk strains, or foreign-plant introductions were included. Such strains have been propagated as composite lots and do not represent single plants. When seedlings were 2–3 inches tall, the sixteen to twenty-four

plants taken from each lot were re-established individually in small plant bands containing unsterilized soil. Subsequently the seedlings were transplanted into the field in early May, as progenies of the original panicles. Figures 3 and 4 illustrate the method of culture and the general appearance of progenies in early summer of the second year following establishment. Observations were made of the vigor and relative numbers of seedlings produced from each panicle, though these characters will not be considered in this presentation. The plants were usually vigorous, with percentage of survival very high each year. In the fields, progeny rows were 42 inches distant, and the plants were spaced 2 feet apart in the rows.

Observations were made upon relative vigor, uniformity, and disease reaction during late September of the first year. Plants not corresponding to the principal type of the progeny (aberrants) were noted, although first season's growth is not typical of that in later years. Detailed notes were taken periodically in the second season for a number of characters. These included uniformity, early (spring) vigor, early growth habit, aggressiveness, leafiness, leaf width, leaf length, foliage color, num-

FIG. 2.—Distribution of 541 collections of *Poa pratensis* in Wisconsin, 1940–1943



FIGS. 3–4.—*Poa pratensis*. Fig. 3 (*above*), relatively vigorous progenies in rows early in second year of growth. Fig. 4 (*below*), progeny rows showing variations in color, vigor, and growth habit. Note highly sexual type on left. Consistent change of type in row indicates another progeny. Much of color difference is due to mildew infection.

ber and size of culms, plant height, earliness of seed maturity, reaction to diseases, and late (fall) vigor. Not all these characters will be considered in the present paper, though they were of use in determining the percentages of aberrant types. Data on early vigor were secured in 1943 only and represent only a part of the progenies.

The following tabulation summarizes the notes obtained concerning the morphological characters analyzed. Differences were indicated by the assignment of number values representing the relative development of these characters among plants and progenies. These number values are employed in several of the tables.

Early vigor. Lowest number most vigorous.

Growth habit. This character is based upon the general profile form of the plant and is somewhat related to aggressiveness: 1, prostrate; 2, near prostrate; 3, semiprostrate; 4, near-tussock; 5, tussock.

Aggressiveness. Lowest number most aggressive.

Leafiness. Lowest number most leafy.

Maturity. E, early; M, medium; L, late.

Late vigor. Lowest number most vigorous.

ALLISON and CHAMBERLAIN (3) reported the occurrence on bluegrass of the diseases studied, and the former author verified the identification of the causal organisms in subsequent years.

Results

ABERRANT FREQUENCY

UNITED STATES.—The breeding behavior of the 800 collections from different regions of the northern United States is shown in table 1. The large number of collections for the Central Lowland includes the Wisconsin lots later considered separately. The classification into physiographic regions is a modification of that of FENNEMAN (9).

TABLE 1

BREEDING BEHAVIOR OF POA PRATENSIS LOTS COLLECTED IN DIFFERENT REGIONS OF THE UNITED STATES WHEN GROWN AT MADISON, WISCONSIN

REGION	NO. OF COLLECTIONS	SEGREGATING		AV. % ABERRANTS BASED UPON—		MAX. % ABERRANTS
		No.	%	Total	Seg.	
Northeastern	35	31	88.6	26.2	29.6	60.0
Central Lowland	577	423	73.3	14.9	20.4	100.0
East Central	13	11	84.6	24.1	28.5	75.0
Northern Plains	35	24	68.6	11.7	17.1	56.0
Central Plains	45	39	86.7	17.0	19.6	44.0
Rocky Mountains	70	64	91.4	16.0	17.5	61.0
Northern Great Basin	9	7	77.7	9.1	11.7	31.0
North Pacific	16	7	43.8	14.7	33.6	38.0

Of the total number of collections, 606 (75.8%) produced segregating progenies. No attempt was made to subject the regional data to a rigid statistical analysis. The chi-square test indicated a close relation between the number of collections from each region and the number segregating, the average percentage reproducing apomictically being, therefore, reasonably similar in the several regions. The data for the North Pacific region may be an exception. Strains from the Rocky Mountain area were highest in the frequency of aberrants; those from

the Northeastern, Central Plains, and East Central sections were the most similar in this respect.

The average percentage of aberrant types for all regions was 16.5. Considering only those families developing aberrants, this percentage was 20.5. It should be stated, however, that these percentages are based upon certain morphological deviations in type, those classified as questionable not being included. The latter amounted to approximately 13.6% of the values given. It appears, therefore, that the percentages may be considered conservative. Significant differences appear to exist among the average percentages of aberrants in strains from the different regions. Progenies from the North Pacific, Northeastern, and East Central areas were generally higher in frequency of aberrants. Although such differences may be real, the chi-square test indicated that variances for the several regions were not homogeneous and that the significance of differences could not be determined on a general basis. Wide ranges in the maximum percentage of aberrant types are indicated, with larger groups tending to exhibit highest values, as might be expected.

TABLE 2

BREEDING BEHAVIOR OF POA PRATENSIS LOTS COLLECTED FROM DIFFERENT SOIL TYPES IN WISCONSIN

SOIL AREAS	NO. OF COLLECTIONS	SEGREGATING		AV. % ABERRANTS BASED UPON—		MAX. % ABERRANTS
		No.	%	Total	Seg.	
Knox* (silt loams and loams)	131	85	65	12	18	100
Miami and Carrington† (silt loams and loams)	97	72	74	18	24	100
Miami and Carrington‡ (sands and sandy loams)	13	9	69	19	13	63
Superior§ (clays and clay loams; northern area)	39	26	67	11	17	75
Superior‖ (clays and clay loams; southern area)	37	30	81	15	18	52
Sands¶	102	68	67	10	15	57
Spencer	41	27	66	12	18	67
Kennan** (silt loams and loams)	56	40	71	12	16	67
Kennan†† (sands and sandy loams)	12	10	83	13	16	50
Peat	5	1	20	5	6	25
Wet land	8	4	50	1	30	6

* Unglaciated region of southwestern Wisconsin characterized by occurrence of Fayette, Dubuque, Gale, Tama, and Dodgeville series.

† Glaciated region of eastern Wisconsin; Carrington includes Parr series principally but with some Carrington.

‡ Glaciated area of northeastern Wisconsin; characterized by occurrence of Onaway, Trenary, and Kewaunee series.

§ Ontonagon series.

‖ Superior and Kewaunee series.

¶ Coloma, Plainfield, and Boone series.

** Includes Kennan, Milaca, Cloquet, and Iron River series.

†† Includes Vilas, Omega, and Hiawatha series.

WISCONSIN.—An analysis is given in table 2 of the percentages of collections producing aberrant types and of the average percentages of aberrants for different soil-type groups in Wisconsin. The classification of soil areas is general and follows that of WHITSON (16). This classification is broad and regional rather than specific, as the individual soil types of the sites at which panicles were collected were not identified. The chi-square test resulted in a very high p value, indicating that relative numbers of progenies segregating were similar for the various

soil areas. The data suggest that collections from peat soil and other wet land are less likely to produce aberrant types than are those from other soil areas, although the number of samples is very small. It is problematical whether differences occurring among collections from other soil areas are significant. The highest percentage of segregating collections is shown for Kennan sands and sandy loams; the next highest is for Superior clays and clay loam (southern area).

A chi-square test of the variances for the percentages of segregation within progenies from the several soil classes showed that the error was heterogeneous. Removal of several selected groups from the analysis did not serve to reduce the chi-square value. The highest average percentage of aberrant plants occurred in collections from wet land; the next highest was obtained for Miami and Carrington silt loam and loam regions, where an average of 24% aberrants based upon the segregating collections was obtained. Although there is some variation in the data for the several soil areas, no obvious relationships between soil and percentage of segregation are apparent. A wide range exists in maximum percentage of aberrants among soils of different classes. Maxima tend to be higher where larger numbers of collections were available, but the data for sandy soils are somewhat divergent from this tendency. It would seem that the ultimate maxima for any soil areas might be similar but that the frequency of such maxima might, for various reasons, be considerably different.

Table 3 contains data with the collections classified according to the length of the growing season where they were obtained. The divisions follow those given in *Climate and man* (7). The chi-square test indicated a very good fit of the classes segregating in proportion to their total numbers. With the exception of the class in the growing season of 90–100 days, they are essentially similar in the percentages of the lots segregating. In this particular class the number of collections was very small, and the percentage is, therefore, relatively unreliable.

Wide differences are shown among the percentages of aberrants based upon the total number of collections. The 90–100-

TABLE 3

BREEDING BEHAVIOR OF POA PRATENSIS LOTS COLLECTED IN WISCONSIN AND CLASSIFIED ACCORDING TO LENGTH OF GROWING SEASON

Class	Av. growing season (days)	No. of collections	Segregating		Av. % aberrants based upon—		Max. % aberrants
			No.	%	Total	Seg.	
1	90–100	3	3	100	39	39	53
2	100–110	14	10	71	8	11	50
3	110–120	61	42	69	9	13	77
4	120–130	104	68	65	11	16	75
5	130–140	47	31	66	9	14	50
6	140–150	137	97	71	16	23	80
7	150–160	120	81	68	12	18	100
8	160–170	36	25	69	14	20	56
9	170–180	22	16	73	17	23	100

day class is again the most divergent, though numbers are too small for significance. With this exception, however, the strains derived from areas with longer growing seasons seemingly have somewhat higher average percentages of aberrants. This relationship is less obvious when the percentages of aberrants among segregating types are considered. The test for homogeneity showed the variance to be heterogeneous in this group. Exclusion of classes 1 and 9, however, reduced the chi-square value to an insignificant amount. The *F* values in the subsequent analysis of variance indicated that significant differences existed between certain percentages. Calculation of the least significant differences for the numerous possible paired comparisons showed 140–150-day lots to be significantly higher in average percentage of aberrants than the 100–110-, 110–120-, 120–130-, 130–140-, and 150–160-day lots. In general, the groups from areas of longer growing season appear to have higher percentages of plants reproducing sexually. The shortest season class (90–100 days) is an exception, but the number of progenies in this group is very small. Relationships among different regions for maximum percentages of aberrants indicate that the maximum is higher, as expected, for the classes with the larger numbers of collections, though some exceptions occur.

Data based upon the classification of collections according to geographic areas of Wisconsin are given in table 4. These areas are from the differentiations made by MARTIN (10). In general, a close similarity is indicated among different regions with respect to the percentages of the collections segregating. Since the chi-square test indicated variance to be homogeneous, an analysis of variance was made.

TABLE 4

BREEDING BEHAVIOR OF POA PRATENSIS LOTS COLLECTED IN WISCONSIN AND CLASSIFIED ACCORDING TO GEOGRAPHIC AREAS

GEOGRAPHIC AREA	NO. OF COLLECTIONS	SEGREGATING		AV. % ABERRANTS BASED UPON—		MAX. % ABERRANTS
		No.	%	Total	Seg.	
Eastern Ridge and Lowlands	103	79	77	16	21	100
Central Plains	103	70	68	11	15	63
Western Upland	192	128	67	14	21	100
Northern Highland	107	73	68	11	16	100
Lake Superior Lowland	42	28	67	11	17	75

P exceeds 0.05; variance between regions, 719.925.

The significantly different paired comparisons for the frequency of aberrants indicate that the Western Upland exceeds in frequency of aberrants the Central Plains and Northern Highland areas and that the Eastern Ridge and Lowlands similarly exceeds the Northern Highland region. The data show that the Eastern Ridge and Lowlands and the Western Upland areas average higher than other geographical regions in percentages of aberrants. This suggests that collections from these areas give progenies somewhat higher in percentages of aberrants based upon the total number and also upon the number of segregating

TABLE 5

NUMERICAL FREQUENCIES OF PERCENTAGE CLASSES OF ABERRANT PLANTS IN STRAINS OF POA PRATENSIS BASED UPON SOIL AREA, LENGTH OF GROWING SEASON, AND GEOGRAPHIC AREA WHERE COLLECTED IN WISCONSIN

CLASSIFICATION	CLASSES FOR PERCENTAGE OF ABERRANT PLANTS											
	0	1–10	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90	91–100	Total
Soil areas*												
Knox (silt loams and loams)	46	33	27	12	3	7	....	....	....	....	2	131
Miami and Carrington (silt loams and loams)	25	25	15	9	10	8	....	2	2	....	1	97
Miami and Carrington (sands and sandy loams)	4	2	4	....	1	....	1	1	....	....	....	13
Superior (clays and clay loams; northern area)	13	9	10	3	3	....	....	....	1	....	....	39
Superior (clays and clay loams; southern area)	7	10	11	4	3	1	1	....	....	....	....	37
Sands	34	29	28	3	1	3	4	....	....	....	....	102
Spencer	14	11	6	6	3	....	....	1	....	....	....	41
Kennan (silt loams and loams)	16	19	12	4	1	2	1	1	....	....	....	56
Kennan (sands and sandy loams)	2	4	4	1	....	1	....	....	....	....	....	12
Peat	4	1	....	....	....	....	....	....	....	....	....	5
Wet land	4	1	1	1	....	....	....	....	1	....	....	8
Total	169	144	118	43	25	22	7	5	4	....	3	540
Length of growing season												
90–100 days	....	....	1	....	....	....	2	....	....	....	....	3
100–110 days	4	8	1	....	....	1	....	....	....	....	....	14
110–120 days	19	20	19	1	....	1	....	....	1	....	....	61
120–130 days	36	28	23	10	4	....	1	1	1	....	....	104
130–140 days	16	16	10	2	1	2	....	....	....	....	....	47
140–150 days	40	30	25	14	9	13	3	2	1	....	....	137
150–160 days	39	33	27	10	3	3	2	1	1	....	1	120
160–170 days	11	5	11	3	4	1	1	....	....	....	....	36
170–180 days	6	6	3	3	3	....	....	....	....	....	1	22
Total	171	146	120	43	24	21	9	4	4	....	2	544
Geographic areas												
Eastern Ridge and Lowlands	23	31	22	11	10	3	2	....	1	....	1	104
Central Plains	29	33	27	7	0	4	1	2	....	....	....	103
Western Upland	65	40	39	18	9	14	3	2	1	....	1	192
Northern Highland	35	35	22	7	3	1	2	....	1	....	1	107
Lake Superior Lowland	14	10	13	1	3	....	....	....	1	....	....	42
Total	166	149	123	44	25	22	8	4	4	....	3	548

* Cf. table 2.

collections than do those from other regions. Maximum percentages of aberrants appear to be reached, as previously indicated, when larger numbers of collections are involved, although the Central Plains area is an exception.

The relative frequencies of percentage classes of aberrant plants for the different soil groups of Wisconsin (**16**) are shown in table 5. The low and zero percentage classes contain most of the collections for all soil areas. There appears to be some tendency for collections from Miami and Carrington silt loam and loam soils to range into somewhat higher classes. Lots from Knox and sandy soils showed somewhat higher frequencies than average in the lower percentage classes. A less marked trend is indicated for collections from certain other soil types to show irregular tendencies. This is particularly notable for lots from Miami and Carrington and Kennan soils.

The frequencies of percentage classes of aberrant plants based upon classification for length of the growing season are also given in table 5. There is an apparent tendency for lots collected from regions having 120–130-, 140–150-, and 150–160-day growing seasons to have somewhat higher frequencies of aberrants than the average. The general relation of the length of the growing season to the frequency of aberrants, however, indicates no particularly striking tendencies.

Data for classification based upon geographic areas in table 5 suggest that lots from the Eastern Ridge and Lowlands and Western Upland areas have somewhat more widely distributed frequencies than the average in the various classes of aberrant plant percentage; the Western Upland area had a somewhat higher proportion in the zero class. Other groups are generally similar to one another in relative distribution.

Growth characters

United States.—The numerical frequencies shown in table 6 are of the United States collections classified for early and late vigor, growth habit, aggressiveness, leafiness, and maturity. Graphical examinations have been made of the frequency distributions of the percentages in the various numerical classes considering each region for each character. These studies indicated that the distributions are essentially similar among all regions for each trait observed. This is particularly true for early vigor, growth habit, and aggressiveness. Comparisons of the relationships among regions for leafiness, maturity, and late vigor were of greater contrast. Only character relationships which were somewhat variable among regions will be discussed.

There was some indication that strains from the Northern Great Basin were more often of the low prostrate growth habit than were those from other regions. The number of samples for this region, however, is quite small. Collections from the East Central region showed a slightly different distribution for aggressiveness than those from other regions, the former being generally less aggressive. Collections from the Northeastern and North Pacific regions were somewhat exceptional to the general average of the material from other regions for leafiness characters; much higher percentages of these two groups were leafy, with the stemmy types being relatively infrequent. In relative maturity the lots from the Northern Plains and the Northern Great Basin were generally earlier than those of other regions from which most collections were classified as medium late to late. Differences in this respect were quite marked. The most diversity occurred with respect to the degree of vigor shown by collections from different

regions at the time of maturity. Material from the Northern Plains and from the Central Plains appeared to be generally more vigorous than that from other regions. Very few lots were classified as extremely low in late vigor.

The numbers of collections from the several regions are quite different, and the relative representation is not entirely comparable. It is concluded, however, that there appears to be little characteristic regional diversity among strains for early vigor, growth habit, and aggressiveness but that there is some contrast among regional lots in leafiness, relative maturity, and late vigor. Whether such

TABLE 6

NUMERICAL FREQUENCIES OF STRAINS OF POA PRATENSIS FROM VARIOUS REGIONS AND STATES IN CLASSES FOR VIGOR, GROWTH HABIT, AGGRESSIVENESS, LEAFINESS, AND MATURITY

REGION AND STATE	EARLY VIGOR				GROWTH HABIT					AGGRESSIVENESS			LEAFINESS			MATURITY			LATE VIGOR			
	1	2	3	4	1	2	3	4	5	1	2	3	1	2	3	E	M	L	1	2	3	4
1. Northeastern:																						
Maine	...	5	5	...	...	...	4	3	3	...	7	3	5	2	3	3	1	6	1	3	4	2
New York	1	2	...	...	...	...	2	...	1	1	1	1	1	2	...	...	1	2	1	1	1	...
Pennsylvania	...	5	3	...	...	...	5	3	...	1	4	3	5	3	...	...	...	8	2	4	2	...
Rhode Island	1	6	7	...	...	2	6	5	1	1	12	...	12	1	1	...	1	11	5	5	3	1
Total	2	18	15	...	...	2	17	11	5	3	24	7	23	8	4	3	3	27	9	13	10	3
2. Central Lowland:																						
Illinois	1	1	1	...	...	1	1	1	...	1	2	...	1	2	...	...	...	3	3	...	...	...
Indiana	1	1	...	...	...	...	1	1	...	1	1	...	2	...	...	...	2	...	2	...	...	...
Iowa	...	5	4	...	1	3	3	2	...	...	5	4	2	6	1	...	2	7	4	2	3	...
Kentucky	2	8	1	...	...	2	5	3	1	1	9	1	4	6	1	...	3	8	6	4	...	...
Michigan	1	6	3	...	...	...	5	4	1	3	5	2	5	4	...	...	1	9	5	3	2	...
Minnesota	6	20	10	...	...	15	11	7	3	4	28	4	16	18	2	...	6	19	19	14	3	...
Missouri	3	7	1	...	2	2	6	...	1	1	9	1	6	5	...	...	4	7	4	6	1	...
Ohio	10	11	5	...	1	3	12	8	2	8	14	3	17	8	1	...	6	20	14	9	2	...
Wisconsin	20	65	41	...	10	30	55	19	11	18	69	39	36	70	20	3	54	69	43	53	23	6
Total	44	124	66	...	14	56	99	45	19	37	142	54	89	119	25	3	78	142	100	97	34	6
3. East Central:																						
North Carolina	...	6	2	...	...	2	3	2	1	...	1	7	2	6	...	...	5	3	5	3	...	...
Virginia	...	...	1	...	...	...	1	...	...	...	1	...	1	...	...	...	...	1	1	...	...	...
West Virginia	2	...	2	...	...	...	1	1	2	1	2	1	1	3	...	...	2	2	3	1	...	...
Total	2	6	5	...	...	2	5	3	3	1	4	8	4	9	...	...	7	6	9	4	...	...
4. Northern Plains:																						
Nebraska	7	20	3	...	5	7	11	4	3	8	21	1	5	20	5	...	20	10	21	9	...	...
North Dakota	1	3	...	...	...	1	3	...	...	...	3	...	1	2	...	...	2	2	2	1	1	...
South Dakota	...	1	...	...	...	...	...	...	1	...	1	...	1	...	...	...	1	...	1	...	...	...
Total	8	24	3	...	5	8	14	4	4	8	25	1	7	22	5	...	23	12	24	10	1	...
5. Central Plains:																						
Arkansas	1	5	1	...	1	...	3	1	2	1	4	2	3	3	1	...	3	4	2	5	...	...
Kansas	4	17	9	...	...	6	19	4	1	1	26	3	7	20	3	...	6	24	12	12	5	1
Oklahoma	4	3	1	...	...	2	1	3	2	...	7	1	6	2	...	...	2	6	1	3	3	1
Total	9	25	11	...	1	8	23	8	5	2	37	6	16	25	4	...	11	34	15	20	8	2
6. Rocky Mountains:																						
Colorado	2	8	...	...	...	2	5	2	1	...	8	2	5	5	...	...	4	6	8	1	1	...
Montana	1	3	2	...	1	1	3	...	1	2	3	1	...	6	...	...	5	1	5	1	...	...
Utah	...	10	...	...	1	4	4	1	...	6	4	...	3	2	5	1	4	5	4	2	...	...
Total	3	21	2	...	2	7	12	3	2	8	15	3	8	13	5	1	13	12	17	4	1	...
7. Northern Great Basin:																						
Idaho	...	3	1	...	...	3	1	...	...	1	2	1	...	3	1	...	3	1	3	1	...	...
Oregon	...	1	1	...	...	1	1	...	...	...	2	...	...	2	...	...	2	...	1	1	...	...
Washington	...	1	2	...	...	...	1	2	...	...	1	2	...	3	...	2	1	...	1	2	...	...
Total	...	5	4	...	...	4	3	2	...	1	5	3	...	8	1	2	6	1	5	4	...	...
8. North Pacific:																						
Washington	2	7	7	...	3	2	9	1	1	7	7	2	9	7	...	...	4	11	2	9	5	...

differences are significant with respect to the natural development of regional types of bluegrass appears to be questionable.

WISCONSIN.—Numerical frequencies are presented in table 7 for the soil, growing season, and geographic classifications of the Wisconsin collections for the several characters of growth. It is somewhat difficult to make comparisons because of insufficient numbers in some instances. These frequencies are not directly comparable with those for the collections for the entire United States. Much diversity was shown with respect to soil areas.

Considerable diversity in frequencies for growth habit existed among the col-

TABLE 7

NUMERICAL FREQUENCIES OF STRAINS OF POA PRATENSIS IN CLASSES FOR VIGOR, GROWTH HABIT, AGGRESSIVENESS, LEAFINESS, AND MATURITY WHEN BASED UPON SOIL AREA, LENGTH OF GROWING SEASON, AND GEOGRAPHIC AREA WHERE COLLECTED IN WISCONSIN

CLASSIFICATION	EARLY VIGOR			GROWTH HABIT					AGGRESSIVENESS				LEAFINESS				MATURITY			LATE VIGOR				
	1	2	3	1	2	3	4	5	1	2	3	4	1	2	3	4	E	M	L	1	2	3	4	5
Soil areas*																								
Knox (silt loams and loams)	8	19	14	14	39	35	12	3	14	36	23	32	25	61	18	0	26	45	34	14	41	35	12	3
Miami and Carrington (silt loams and loams)	3	6	8	4	21	20	15	1	9	16	24	10	23	25	11	1	21	22	18	6	11	17	23	3
Miami and Carrington (sands and sandy loams)	1	8	3	1	2	4	3	2	2	6	5	0	2	9	3	0	1	5	7	0	3	4	4	2
Superior (clays and clay loams; northern area)	0	3	2	6	7	7	14	5	9	14	4	13	15	17	5	2	23	7	9	1	3	8	20	7
Superior (clays and clay loams; southern area)	0	0	0	2	2	0	0	0	0	1	0	3	0	1	3	0	2	2	0	0	1	0	2	1
Sands	7	15	6	11	28	37	9	5	11	35	26	23	17	42	16	15	42	36	12	6	21	29	31	5
Spencer	1	4	2	3	7	13	16	2	9	6	15	11	24	12	2	3	18	9	15	3	7	13	16	2
Kennan (silt loams and loams)	1	0	0	1	8	18	7	1	3	2	16	14	10	16	6	3	19	12	4	1	2	13	16	3
Kennan (sands and sandy loams)	0	4	1	0	5	2	0	2	0	5	3	1	1	5	3	0	3	5	1	0	5	2	0	2
Peat	0	0	0	0	0	3	1	1	0	2	3	0	1	2	1	1	5	0	0	0	0	3	1	1
Wet land	1	2	0	0	1	3	2	1	1	4	1	1	2	7	4	1	5	0	3	1	1	3	2	1
Total	22	61	36	42	120	142	79	23	58	127	120	108	120	197	72	26	165	143	103	32	95	127	127	30
Length of growing season																								
90–100 days	0	0	0	0	1	2	0	0	0	2	0	1	0	2	1	0	1	1	1	0	2	0	1	0
100–110 days	0	0	0	0	2	8	3	1	1	1	5	7	6	7	1	0	8	4	2	1	0	6	5	2
110–120 days	3	4	1	13	22	17	5	3	3	15	26	17	12	29	12	8	18	27	16	5	7	15	31	3
120–130 days	0	6	4	7	20	30	34	7	17	26	25	29	37	33	18	8	25	40	31	17	19	30	27	4
130–140 days	3	6	6	3	13	20	9	3	5	15	18	10	12	18	12	6	8	27	13	6	15	16	11	0
140–150 days	5	18	9	4	7	32	14	5	12	22	25	3	21	28	9	4	25	19	18	11	21	20	8	2
150–160 days	7	15	6	12	36	27	7	3	13	28	18	29	15	53	15	0	27	32	26	13	15	16	39	2
160–170 days	4	13	9	4	11	8	4	3	3	17	6	4	8	18	4	0	2	10	18	7	11	5	4	1
170–180 days	1	1	3	1	7	2	4	0	1	2	10	1	4	6	3	0	6	2	6	2	1	3	8	0
Total	23	63	38	44	119	146	80	25	55	128	133	101	115	194	75	26	120	162	131	62	91	111	134	14
Geographic areas																								
Eastern Ridge and Lowlands	4	16	4	5	25	21	16	2	12	23	25	15	25	30	15	0	18	21	29	13	18	16	27	4
Central Plains	7	8	7	11	23	40	12	7	6	19	40	28	22	47	22	13	21	50	22	10	18	30	35	
Western Upland	8	31	23	14	43	39	13	4	15	50	24	24	24	73	15	1	21	39	53	18	39	22	32	1
Northern Highland	2	5	2	9	21	36	26	7	14	23	29	34	34	46	20	10	32	37	31	7	19	32	35	7
Lake Superior Lowland	0	5	2	6	7	11	14	4	8	15	6	13	13	20	7	2	9	19	14	15	6	11	8	2
Total	21	65	38	45	119	147	81	24	55	130	124	114	118	216	79	26	101	166	149	63	90	111	137	14

* Cf. table 2.

lections from the various soil classes, but few of these appeared to have any notable characteristics. The classification for aggressiveness showed considerable variation among lots. Those from Spencer and Kennan silt loam and loam areas generally had somewhat similar frequencies; those from the two Superior clay and clay loam areas had similar distributions, with a tendency toward nonaggressive types. Other than these generalizations, the relationships were sufficiently irregular as to appear to be of little significance.

In the frequency distribution of collections classified for leafiness, leafy types were relatively more numerous in the data for the Spencer soil area than for any of the other soil groups; otherwise the frequency distributions for leafiness classes were quite similar.

Frequencies for maturity classes showed greater divergence among collections from different soil areas than did those for other characters. Lots from peat soil were notably early in maturity. Those from Kennan silt loams and loams, Kennan sands and sandy loams, wet land, and Superior clays and clay loams were also relatively early in maturity.

Types from Kennan sand and sandy loam soils had a high frequency of vigorous strains in the classification for late vigor. This is of doubtful significance. A relatively high frequency of marked vigor was also shown by lots from wet land. A relatively high frequency of moderate to poor late vigor was indicated for types derived from Superior clays and clay loams. Lots from Spencer soil and Kennan silt loam and loam soils showed somewhat similar distributions but had relatively higher frequencies in the medium vigorous and vigorous classes.

Frequency comparisons for classification based upon length of growing season (table 7) show that the relationships among classes for early vigor, growth habit, and leafiness are fairly comparable, no marked differences in regions being indicated. The collections from areas of shorter growing seasons showed relatively high percentages of intermediate types with respect to growth habit, whereas the 160–170- and 170–180-day regions presented a somewhat disproportionate frequency of low-growing types. Collections from the 140–150-day region tended to resemble those from the areas of shorter growing seasons.

Distributions for the seasonal areas based upon aggressiveness were quite irregular. Relatively more aggressive types were found in the 90–100-day region; a fairly high percentage of aggressive types was also shown in the 160–170-day region. The frequency of such types was low in the 170–180-day region, and the 100–110-day region showed an unexpectedly high proportion of nonaggressive types. Distributions of frequencies in leafiness classes for the various lengths of growing seasons were very similar, no notable tendency being indicated.

Classification for maturity presented the most irregular data of any of the characters studied, no relationship appearing to exist among regions for this character. There was a tendency for lots from the 100–110-day region to be early in maturity. Collections from the 110–120-day area showed no apparent relationships. Lots from the 160–170-day area were relatively late in maturity, but those from the 170–180-day area showed no apparent relationship to the groups from the areas of shorter season.

The distributions of frequencies in the classification for late vigor were generally quite different for the various seasonal groups. Lots from the 90–100-day group showed a relatively high percentage of

progenies of marked vigor, but those from the 100–110- and 110–120-day groups exhibited a general tendency to lack vigor, as did those from the 150–160 and 170–180 groups. Data for the 120–130, 130–140, and 140–150 groups present fairly comparable distributions, with some tendency for types to be average in vigor. The 160–170-day class was higher than average in vigor. It should be noted in the classifications for growing season that only three collections were represented in the 90–100-day class.

In summary it may be said that grouping the collections on the basis of the average lengths of the frost-free season indicates much variation among groups with respect to the characters studied but no consistent relationships. Most diversity exists in the characters of aggressiveness, maturity, and late vigor, and it is very difficult to see any general trends.

When the data were grouped by geographic origin of collections (table 7), the frequency distributions among the early vigor classes were very similar for the several areas except for the Central Plains region, where fewer intermediate types occurred. In growth habit the Eastern Ridge and Lowlands and the Western Upland areas showed proportionately higher numbers of low-growing types, while the Lake Superior Lowland group had a proportionately higher frequency of the bunch type. Considerable diversity was shown among regions in the classes for aggressiveness; only the Western Upland and Lake Superior Lowland distributions were closely similar. The latter area showed a proportionately large number of nonaggressive types, as did the Northern Highland group. The Central Plains class was relatively high in intermediate types. Distributions for frequencies of classes for leafiness were very similar among the regions with the Western Upland group containing more intermediate types. In the maturity classification the Eastern Ridge and Lowlands and the Western Upland classes were similar in their relative frequencies. The other three areas were somewhat alike among themselves, but the Northern Highland group contained proportionately fewer intermediates. Classification for late vigor showed much irregularity in relation to geographic areas. The Eastern Ridge and Lowlands, the Central Plains, and the Northern Highland groups were not widely divergent. Lots from the Western Upland and Lake Superior Lowland regions were divergent and between themselves showed somewhat opposite frequencies except for the least vigorous classes.

Among the several characters analyzed the variation in leafiness was most comparable, whether groups were based upon soil areas, upon relative lengths of the growing season, or upon geographic sections in Wisconsin. Early vigor and growth habit might be considered next most constant, whereas aggressiveness and late vigor were most variable.

Classification for soil areas produced most irregularity in relative frequencies, while grouping based upon geographic areas showed least diversity.

Plant height

United States.—Although plant height at maturity is perhaps a character of little practical importance, it seemed evident that its value might be more accurately measured than other plant features. A comparison of strains upon this basis for the geographic regions of the United States is presented in table 8, although the number of strains was generally insufficient to permit detailed comparisons of frequencies in the differ-

ent regions. The collections from the Northeastern and the Central Lowland sections of the country perhaps include types averaging somewhat shorter in culm length. Distribution of the lots from the Central Lowland also suggests a negative skewness compared with the average frequencies of all progenies. It would appear very doubtful whether the differences indicated are of any significance.

WISCONSIN.—Collections from the various soil areas of Wisconsin have been classified for culm height (table 9). Inage type. Strains from the 150–160-day area included an exceptional number of medium short types; a two-peaked curve resulted.

Data for geographic areas (table 9) suggest that all distributions resemble the normal, though minor variations occur. Western Upland and Northern Highland strains included proportionately more medium short types, while the Eastern Ridge and Lowlands and Northern Highland areas had relatively numerous tall biotypes.

TABLE 8

NUMERICAL FREQUENCIES FOR HEIGHT OF PLANT OF STRAINS OF POA PRATENSIS FROM GEOGRAPHIC REGIONS OF THE UNITED STATES

REGION	PLANT HEIGHT IN INCHES AT MATURITY																				
	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	Total
Northeastern	1	1	1	...	3	3	4	4	3	8	2	1	1	1	1	...	1	...	...	...	35
Central Lowland	...	1	2	6	3	3	7	18	17	26	38	38	21	19	10	7	4	11	1	3	235
East Central	...	...	...	...	...	...	...	...	2	2	...	2	2	4	1	...	...	...	...	...	13
Northern Plains	...	...	...	...	...	3	...	2	1	2	3	6	7	7	1	2	...	...	...	...	34
Central Plains	...	...	...	...	1	3	3	3	5	9	4	5	4	5	2	...	1	...	...	...	45
Rocky Mountains	...	...	...	...	...	...	3	...	1	1	2	6	2	6	2	1	1	1	...	...	26
Northern Great Basin	...	...	...	...	...	...	1	...	1	...	...	2	2	2	...	1	...	...	...	...	9
North Pacific	1	...	...	...	1	1	...	2	...	...	3	2	2	1	1	2	...	...	...	...	16
Total	2	2	3	6	8	13	18	29	30	48	52	62	41	45	18	13	7	12	1	3	413

sufficient information is available to make comparisons between some regions, and data are meager for all soil regions. Comparing relative frequencies, distributions are somewhat similar. Progenies from plants of Spencer soils and of Kennan silt loams and loams were somewhat deficient in types of average height and were more variable than the average.

Results of the classification based on the length of the growing season are also presented in table 9. The 100–110- and 130–140-day classes were more variable than the other groups with proportionately more short and more tall types. Other distributions approached the aver-

Groupings based upon length of the growing season appeared to show more diversity in plant height than those based upon soil and geographic areas.

DISEASE REACTIONS

Certain diseases of Kentucky bluegrass occurred with sufficient intensity and general distribution to permit observation of strain reactions. The diseases were not equally abundant in all seasons, and plant reactions were somewhat variable from year to year. The diseases observed included leaf rust caused by *Puccinia poae-sudeticae* (Westend.) Jørstad and *P. rubigovera* (DC.) Wint.;

TABLE 9

NUMERICAL FREQUENCIES OF STRAINS OF POA PRATENSIS IN CLASSES FOR HEIGHT OF PLANT WHEN BASED UPON SOIL AREA, LENGTH OF GROWING SEASON, AND GEOGRAPHIC AREA WHERE COLLECTED IN WISCONSIN

Classification	Culm height in inches at maturity																			
	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	Total
Soil areas*																				
Knox (silt loams and loams)	...	1	2	1	4	5	3	14	7	14	16	12	11	5	7	1	2	...	...	105
Miami and Carrington (silt loams and loams)	...	...	...	...	...	1	...	1	...	...	...	...	...	...	...	...	...	...	...	2
Miami and Carrington (sands and sandy loams)	...	1	4	...	2	...	1	3	8	11	6	5	6	6	2	1	...	...	...	56
Superior (clays and clay loams; northern area)	...	...	...	1	...	...	2	1	1	1	1	2	...	...	1	1	2	...	...	13
Superior (clays and clay loams; southern area)	...	1	...	...	...	...	1	1	1	3	3	3	...	2	...	...	1	...	...	16
Sands	...	...	...	...	...	...	1	...	...	...	...	...	2	1	...	...	...	...	...	4
Spencer	1	...	1	...	2	...	3	4	8	9	4	4	5	9	3	4	2	...	1	60
Kennan (silt loams and loams)	...	...	...	...	2	2	1	5	4	2	5	3	2	1	3	3	...	...	...	33
Kennan (sands and sandy loams)	...	...	3	1	2	2	4	4	3	2	...	...	1	...	1	...	1	...	1	25
Peat	...	...	2	...	...	...	...	...	...	1	2	1	2	...	...	1	...	...	...	9
Wet land	...	...	...	...	...	...	1	...	...	...	1	...	...	...	...	...	1	...	...	3
Total	1	3	12	3	12	10	17	33	32	43	38	30	29	24	17	11	9	...	2	326
Length of growing season																				
90–100 days	...	...	...	...	...	...	...	...	1	...	...	...	...	...	...	...	...	...	...	1
100–110 days	...	...	2	1	1	2	2	2	...	1	...	...	1	...	1	...	...	...	...	13
110–120 days	1	...	1	...	2	...	3	2	6	7	4	5	1	5	2	1	2	...	1	43
120–130 days	...	1	...	1	3	2	1	8	6	7	7	3	4	3	6	4	1	...	...	57
130–140 days	...	...	2	...	1	2	2	3	7	1	2	2	2	1	...	2	2	...	...	29
140–150 days	...	1	2	...	2	1	2	2	7	11	5	3	7	4	1	1	1	...	1	51
150–160 days	...	1	...	1	3	2	6	11	2	7	16	10	10	8	4	5	4	...	...	90
160–170 days	...	...	1	...	...	...	1	2	2	7	4	5	4	3	1	...	...	...	...	30
170–180 days	...	...	4	...	...	...	...	1	1	2	1	2	...	...	2	...	...	...	...	13
Total	1	3	12	3	12	9	17	31	32	43	39	30	29	24	17	13	10	...	2	327
Geographic areas																				
Eastern Ridge and Lowlands	...	1	4	...	1	1	5	3	9	11	7	6	7	9	3	4	3	...	...	74
Central Plains	1	...	3	1	3	1	3	6	8	7	5	5	3	4	3	2	2	...	...	57
Western Upland	...	1	2	...	4	4	4	12	7	15	17	14	13	6	5	1	3	...	1	109
Northern Highland	...	...	2	2	5	3	4	10	5	7	6	3	5	3	7	5	1	...	1	69
Lake Superior Lowland	...	1	1	...	...	...	1	2	2	3	3	3	1	2	...	...	1	...	...	20
Total	1	3	12	3	13	9	17	33	31	43	38	31	29	24	18	12	10	...	2	329

* Cf. table 2.

powdery mildew (*Erysiphe graminis* DC.), and leaf spot (*Helminthosporium vagans* Drechs.). It is thought, however, that *P. rubigovera* was relatively unimportant in the season when leaf-rust reaction was observed. Since data were not obtained for all collections, numbers may not coincide with those for other characters. The plant reactions were recorded numerically; 0, no disease observed; 1, resistant; higher numbers represented progressively a more susceptible reaction. Those classified as of reaction 3 were considered to be very susceptible.

TABLE 10

NUMERICAL FREQUENCIES FOR DISEASE-REACTION CLASSES OF STRAINS OF POA PRATENSIS FROM VARIOUS REGIONS OF THE UNITED STATES

REGION	MILDEW				LEAF RUST			
	0	1	2	3	0	1	2	3
Northeastern	17	3	6	9	13	19	2	1
Central Lowland	41	22	28	17	9	76	17	7
East Central	3	2	5	3	2	10	1	0
Northern Great Plains	4	19	11	0	1	27	4	2
Central Great Plains	18	7	16	4	8	28	8	2
Rocky Mountains	8	8	10	0	3	16	6	3
Northern Great Basin	2	5	3	0	1	5	2	1
North Pacific	4	5	1	2	4	9	0	0
Total	97	71	80	35	41	190	40	16

UNITED STATES.—Numerical frequencies of reaction types for the several regions of the United States are shown in table 10. Insufficient leaf spot developed in 1943 to provide information concerning reaction toward this disease. The frequency distributions for classes of reaction to mildew were very diverse for the several regions; those for the Northern Plains and for the Northern Great Basin were essentially similar, and those for the Rocky Mountain and North Pacific areas varied in a different manner. Other regional distributions comprised a third group. The Northeastern region showed a somewhat higher proportion of mildew-free strains but also a higher number of susceptible ones. Intermediate reaction types were lacking. The highest proportion of susceptible types occurred in the Northern Plains group. Approximately 34% of all collections studied were classified as mildew-free, whereas 12% were very susceptible.

The frequency distributions for leaf-rust classification were very closely similar for all regions. High percentages of the collections were rust-free or were slightly infected, but no regional tendencies were evident.

WISCONSIN.—Data for classification according to soil in Wisconsin are given in table 11. Frequency distributions for mildew reaction were generally similar among the soil groups except those for Kennan sands and sandy loams and for wet land where few data were available. Miami and Carrington silt loams and loams and Kennan silt loam and loam classes included more resistant progenies than the average. Of all the strains, however, 85% were classified as mildew-free or resistant.

Frequency distributions for the several

TABLE 11

NUMERICAL FREQUENCIES OF STRAINS OF POA PRATENSIS IN DISEASE-REACTION CLASSES WHEN GROUPED ACCORDING TO SOIL AREA, LENGTH OF GROWING SEASON, AND GEOGRAPHIC AREA WHERE COLLECTED IN WISCONSIN

Classification	Mildew					Rust					Leaf spot			
	0	1	2	3	4	0	1	2	3	4	0	1	2	3
Soil areas*														
Knox (silt loams and loams)	45	31	9	2	3	5	39	39	21	2	21	28	12	2
Miami and Carrington (silt loams and loams)	36	10	1	3	....	2	24	20	7	1	20	15	3	1
Miami and Carrington (sands and sandy loams)	1	2	2	....	....	1	6	2	3	....	....	....	....	....
Superior (clays and clay loams; northern area)	11	6	3	1	1	....	16	16	5	2	6	14	7	1
Superior (clays and clay loams; southern area)	4	....	....	....	....	....	3	....	1	....	2	2	....	....
Sands	26	24	8	1	....	1	56	27	6	1	17	22	7	5
Spencer	17	15	....	....	1	....	8	15	13	1	7	14	4	5
Kennan (silt loams and loams)	20	4	1	1	1	4	18	9	4	....	5	24	2	2
Kennan (sands and sandy loams)	1	3	3	....	....	2	7	1	....	....	....	....	....	....
Peat	....	....	2	....	....	....	1	1	2	1	....	1	....	....
Wet land	....	4	2	1	....	....	3	4	....	....	....	1	....	....
Total	161	99	31	9	6	15	181	134	62	8	78	121	35	16
Length of growing season														
90–100 days	1	....	....	....	....	....	1	2	....	....	....	2	1	....
100–110 days	10	2	....	....	1	2	7	5	1	....	1	10	2	1
110–120 days	29	7	2	1	1	....	35	21	2	3	19	18	9	1
120–130 days	34	19	4	1	1	5	29	32	24	2	12	42	13	7
130–140 days	13	9	8	3	....	5	28	10	4	....	4	19	1	1
140–150 days	19	11	6	1	....	2	28	16	10	2	9	9	2	1
150–160 days	41	28	6	1	3	5	29	32	24	....	24	24	8	5
160–170 days	4	10	2	....	....	2	23	4	1	....	1	3	....	....
170–180 days	8	....	1	1	....	3	7	4	....	....	6	2	....	....
Total	159	86	29	8	6	24	187	126	66	7	76	129	36	16
Geographic areas														
Eastern Ridge and Lowlands	53	7	2	2	....	7	29	19	15	1	22	18	2	5
Central Plains	45	8	6	3	....	6	39	15	2	1	19	17	1	1
Western Upland	55	37	12	1	3	7	47	36	18	....	15	21	7	2
Northern Highland	47	20	....	....	2	1	17	29	18	3	14	28	8	....
Lake Superior Lowland	19	2	1	....	2	1	9	9	2	1	7	5	1	....
Total	219	74	21	6	7	22	141	108	55	6	77	89	19	8

* Cf. table 2.

soil regions with respect to reaction to rust were very similar and approached a normal distribution. Types from Spencer soils were somewhat more rust-susceptible as a group. Many strains, including 45% of the total, were classified as resistant, while only 4% were noted as rust-free.

Classification for leaf-spot reaction indicated slight differences among soils areas as to behavior of strains. Lots from Kennan silt loams and loams were somewhat more resistant as a group. Approximately 80% of the total number of strains observed were of leaf-spot-free or only slightly infected types. This is essentially the same proportion as that observed for mildew reaction.

Data for classification of disease reaction according to length of the growing season where strains were collected show that general similarity existed among groups in reaction to mildew (table 11). The only exception to this occurred in lots from the 160–170-day area, in which intermediate reaction types were more frequent than in other groups. Classification for rust reaction showed prominent differences among growing-season groups. The 120–130- and 150–160-day classes had distributions tending more toward susceptibility than other groups which were, among themselves, very similar. Most seasonal group distributions approached normality with respect to leaf-spot reaction; that for the 170–180-day season included relatively more leaf-spot-free strains than others.

The disease reactions of strains grouped by geographic regions are given in table 11. Mildew reactions of the several regional classes were quite comparable, those from the Western Upland including a relatively lower proportion of mildew-free strains than the others. Some variations are indicated in the distributions for reaction to rust. They were relatively similar, however. Central Plains lots were somewhat higher in proportion of resistant types than the others, whereas the Northern Highland group was inclined more toward susceptible reactions. Classification for leaf-spot reaction indicated that the Western Upland and Northern Highland areas included proportionately fewer disease-free strains than other sections.

In considering the classification for disease reaction when strains have been grouped according to soil, length of growing season, and geographic areas, it should be noted that numbers of collections are frequently too small to permit critical comparisons. This is particularly evident for leaf-spot reaction.

Frequency distributions of reaction classes for mildew were highly variable for the several regions of the United States and were not normal in type. They were somewhat less variable for the soil and growing-season classifications of Wisconsin data but were not normal, in that populations included excessive percentages of mildew-free and resistant types. Reactions to rust among the various lots from United States and Wisconsin sources were more similar than were reactions to mildew. Very little difference was apparent among the geographic areas of the country. Somewhat more irregularity existed in frequency distributions of data from Wisconsin, particularly with respect to soil groups. Frequency distributions among Wisconsin strains for leaf-spot reaction were approximately normal except for an excess of the resistant types. Considering the diseases observed, there was little indication that the region or the habitat where collected had served to modify the population relative to disease reaction.

Discussion

These studies were an attempt to investigate further the nature of variability in *P. pratensis* and its possible relations to geographical and climatological factors. Though a seemingly large number of progenies have been observed, it is very certain that they cannot be considered as completely representative of the regions sampled. The number of collections made in Wisconsin is perhaps a reasonably adequate sample of that area. Until more extensive data are available, those presented and the generalizations made may contribute to the increasing fund of knowledge about Kentucky bluegrass.

Approximately 75% of all progenies showed some morphological variability. Since numbers of plants grown in each progeny were small, it is probable that many of the types presumed to be constant were potentially variable. The average percentage of aberrant plants was 16.5 when based upon the total number. Considering only those progenies exhibiting segregation, this value was 20.5%. These figures are somewhat higher than those obtained by other workers, though they are reasonably similar to the results presented by BRITTINGHAM (4). It is the view of the writers that they are representative for the collections studied. Thus *P. pratensis* might be described as reproducing generally by apomixis; this was also BRITTINGHAM'S conclusion. Sexual reproduction may play a more important role in maintaining genetic diversity, however, than has been thought previously. Since uniformity is perhaps of little practical advantage for turf and pasture purposes, reasonable conformity to a parental type might suffice for maintenance and utilization of any original biotype selected.

There is some evidence in the results obtained that ecological conditions have served to allow the development of certain regional differences in the general relation of apomictic and sexual reproduction. This is not in agreement with the observations reported by BRITTINGHAM. Whether such differences were generally significant, however, could not be determined. Lowest percentages of aberrants appeared in lots from interior regions in the United States.

Comparisons were made of the relative morphological variability among regional collections. Although minor differences have been indicated, it would appear that regional and local influences have not served to isolate readily recognizable, morphological type-complexes. These results are in accord with those of ÅKERBERG (2), who reported that form-complex studies of *P. pratensis* did not indicate that Eu-*pratensis* was confined to any particular locality. He also observed that the Eu-*pratensis* forms were highly variable and widely scattered. Although the data are not strictly comparable, these results do not appear to support the conclusions of WELLHAUSEN and WEIBEL (15).

Leafiness, late vigor, and earliness of maturity appeared to show more regional differentiation than did other characters in the collections from various parts of the United States. Data for Wisconsin collections indicated that these same characters were highly variable, though leafiness was less so. Early vigor, growth habit, aggressiveness, and plant height did not show prominent regional characteristics. Disease reactions were not of value in differentiating regional classes, though some variation occurred.

Certain general impressions were obtained of progenies growing in the field. Those from the North Pacific and Northeastern regions of the United States ap-

peared to be more leafy, darker green in color, and more generally attractive from a plant-breeding viewpoint. They appeared also to be more generally variable in type within groups. Collections from the Central Plains were relatively less leafy, more prostrate, and more generally similar than those from other sections.

Among collections made in Wisconsin, those from the vicinity of Madison and from the Lake Superior Lowland were most variable and included more attractive types.

It might be concluded that collection of seed of *P. pratensis* from regions differing widely with respect to soil and climatic factors has not greatly increased the probability of obtaining increased variability as compared with more intensive collection in a single locality. Though some evidence contrary to this generalization has been presented, the data obtained are thought to be only suggestive and insufficient to establish this point definitely. When the Wisconsin collections were grouped, particularly on the basis of soil and length of growing season where obtained, greater type diversity was observed.

Summary

1. Eight hundred progenies derived from single panicles of *Poa pratensis* collected in twenty-seven states of the United States were studied in the seasons 1941–1944 at Madison, Wisconsin. These included 541 Wisconsin collections. All were grown in spaced nursery rows.

2. Progenies from the United States were classified according to the geographic region from which the parent seed was collected. Observations were obtained for percentage of aberrant plants, early vigor, growth habit, aggressiveness, leafiness, maturity, late vigor, plant height, and reactions to powdery mildew (*Erysiphe graminis*), leaf rust (*Puccinia poae-sudeticae*), and leaf spot (*Helminthosporium vagans*). Progenies from Wisconsin sources were grouped according to soil area, length of the growing season, and geographic area where parental seed was collected.

3. Among all progenies, 75.8% included some aberrant plants. The average percentage of segregation based upon the total progeny number was 16.5. When only segregating progenies were included, this value was 20.5%.

4. Differences among regions of the United States in percentage of segregation were generally large. No close association of such differences with regional, soil, or climatological variations could be established, although interior or continental regions showed generally lower percentages of aberrant types.

5. Collections grouped by regions of the United States were generally similar to one another in variation of early vigor, growth habit, and aggressiveness. Leafiness, late vigor, and relative maturity varied more extensively among regional groups. Lots from the East Central region were generally less aggressive; those from the Northeastern and North Pacific areas were more leafy; collections from the Northern Plains and the Northern Great Basin were of earlier maturity; and progenies from the Northern Plains and Central Plains were relatively more vigorous.

6. Differences among progenies derived from the various soil areas in Wisconsin were generally small. When grouped according to the length of the growing season in the locality of collection, significant differences in percentages of segregation were found, with the 140–150-day class having a high value. Strains from areas having longer growing seasons were generally somewhat higher in percentages of aberrants than those from areas of shorter seasons. The com-

parison by geographical regions indicated that the Eastern Ridge and Lowlands area and the Western Upland area of Wisconsin were significantly higher in percentages of aberrants than were other areas.

7. Classifications of Wisconsin collections for morphological characters showed a generally great diversity for behavior among lots from different soil areas. Frequency distributions were similar for early vigor and leafiness for the different soils and were somewhat less so for growth habit; those for aggressiveness, maturity, and late vigor were highly dissimilar. Based upon average length of the growing season, distributions for the several characters were generally comparable with those for the soil classification. Frequency distributions for early vigor, growth habit, and leafiness were most similar. The classification for maturity showed least relation to length of growing season. Grouping on the basis of geographical areas resulted in frequencies relatively similar to those for soil and growing seasons. Classification by plant height suggested no particular relationships to region of origin in the United States. In the Wisconsin collections analysis by soil and geographic regions did not indicate prominent differences in relative frequencies of plants with respect to height. When grouped by growing seasons, the 100–110- and 130–140-day lots exhibited relatively lower frequencies of intermediate types, though this was of questionable significance.

8. Collections from the United States varied irregularly in frequency distributions for reaction to powdery mildew. Lots from different regions appeared to differ markedly in proportionate frequencies of resistant and susceptible types. Approximately 34% of the progenies were observed to be mildew-free, whereas 12% were very susceptible. Reaction to leaf rust was generally similar among groups of strains from the various regions of the United States. Of the progenies, 14% were classified as rust-free and 66% as resistant.

9. Classification of Wisconsin collections with respect to soil of origin showed certain prominent differences to exist among soil areas in the character of the distributions among disease-reaction classes. In general, however, distribution for the three kinds of diseases were similar. Considering reaction to leaf rust, collections from certain heavier soil areas were relatively more susceptible to disease than those from others. When classified according to length of the growing season, little relation was shown between length of season and relative frequencies of reaction types, though occasional exceptions occurred. No consistent relation between length of growing season and relative disease resistance was found. Collections grouped according to geographic regions exhibited no significant differences in reaction to diseases.

DEPARTMENT OF AGRONOMY
UNIVERSITY OF WISCONSIN
MADISON, WISCONSIN

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REACTIONS OF DECAPITATED BEAN PLANTS TO CERTAIN OF THE SUBSTITUTED PHENOXY COMPOUNDS[1]

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY **577**

J. M. BEAL

Introduction

The histological reactions of bean plants to certain of the substituted phenoxy compounds applied to the bases of the heart-shaped leaves have recently been described (1). In general, the second internodes of these plants showed tissue responses similar to those induced in the bean by a number of other compounds applied to the cut curface of the young second internode following its decapitation just below the first trifoliate leaf (4, 6). Although all tissues of the second internode, except the epidermis, local areas of the outer cortex, and perhaps the pericycle, proliferated actively as a result of the application of the phenoxy compounds, no roots were developed in this internode during periods as long as 20 days following their application. The responses in the first internode and hypocotyl were somewhat more restricted as to tissues affected than was the case in the second internode. Usually in the first internode and hypocotyl only the endodermis, cambium, phloem, and ray parenchyma responded, but from the cambium, phloem, and ray parenchyma, especially from the two latter, adventitious roots developed in abundance.

Because many of the earlier histological studies had been made following application of the compounds to the cut surface of the second internode of decapitated bean stems, it was decided to apply the phenoxy compounds previously employed (1) to the cut surface of decapitated young second internodes of the same strain of Red Kidney beans as had been used in former experiments. The compounds were applied as 0.5% and

[1] This work was supported in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

as 1% mixtures in both lanolin and Carbowax 1500 as carriers.

Red Kidney bean plants were grown in the greenhouse in flats containing rich loam soil under conditions simulating as closely as possible those used in the former study (1). The second internode was cut squarely across just below the node of the first trifoliate leaf as the leaf started to expand. Immediately afterward the cut surface was covered over with the lanolin or Carbowax mixture, and the plants, together with untreated controls, were kept under conditions favorable for vigorous growth.

Material for histological study was collected at regular intervals after treatment, fixed in Navashin's solution, handled according to the tertiary butyl-alcohol method, and sectioned at 10 μ.

Observations

Gross responses

The gross responses of the decapitated plants to 2-chlorophenoxyacetic, 4-chlorophenoxyacetic, 2,4-dichlorophenoxyacetic (2,4-D), and 2,4,5-trichlorophenoxyacetic acids were, in many respects, different from those resulting from application of these compounds to the bases of the heart-shaped leaves. The visible responses in the decapitated plants were evident chiefly in the upper 10–12 mm. of the second internode near the point of application of the mixtures and in the leaves developed from the axillary buds. All the compounds retarded development of the axillary buds, with the least effects resulting from the application of 2-chlorophenoxyacetic acid, and all the compounds, except 2,4,5-trichlorophenoxyacetic acid, resulted in the formation of at least some dwarfed and often deformed and crinkled leaves on the axillary shoots. No epinasty and little or no stem curvature resulted from the applications.

Under high temperature and humidity the Carbowax mixtures often melted and ran down over the surface of the first internode and sometimes even as far as the upper part of the hypocotyl. Responses in the form of stem enlargement and often of root formation commonly followed. Nearly all the plants, however, to which the Carbowax mixtures were applied showed more or less injury at and near the place of application and frequently rather extensive death of the stem tissues (fig. 1*C*, *E*, *G*). The lanolin mixtures resulted in much less tissue injury (fig. 1*B*, *D*, *F*, *H*).

When Carbowax alone was applied to the apices of decapitated second internodes, slight shrinkage and evidences of partial dehydration of the cells occurred within 24 hours. At 72 hours following application many tiers of dead and shrunken cells were present at the apical ends of the treated internodes (fig. 2). Immediately below these cells was a zone consisting of several tiers of cells in which division had begun. A few layers of these cells adjacent to the dead tissue became suberized during the next few days and functioned as a protective layer over the distal end of the hypocotyl. The volume and area of dead cells resulting from the application of the Carbowax did not vary greatly and did not increase after the first 72 hours in a number of stems which were kept under observation for more than 2 weeks. Injury from the Carbowax alone was in no case as extensive as that from the substituted phenoxy compounds applied in Carbowax.

All the treated plants developed tumor-like swellings at or just below the point of application of the mixtures. The least responses were induced by 2-chlorophenoxyacetic acid (fig. 1*A*, *B*); 4-chloro-

phenoxyacetic (fig. 1*C*, *D*), 2,4-dichlorophenoxyacetic (fig. 1*E*, *F*), and 2,4,5-trichlorophenoxyacetic acids (fig. 1*G*, *H*) showed nearly the same total growth responses. None of the treated plants developed enlargements on the first internodes or hypocotyls except those plants on which the Carbowax mixtures melted and ran down the stems. The time and the rate of elongation of the axillary buds were delayed by all the mixtures, but least by the 2-chlorophenoxyacetic acid. Although the leaves produced by axillary buds on at least a part of all the treated plants showed dwarfing and deformity, except on those to which 2,4,5-

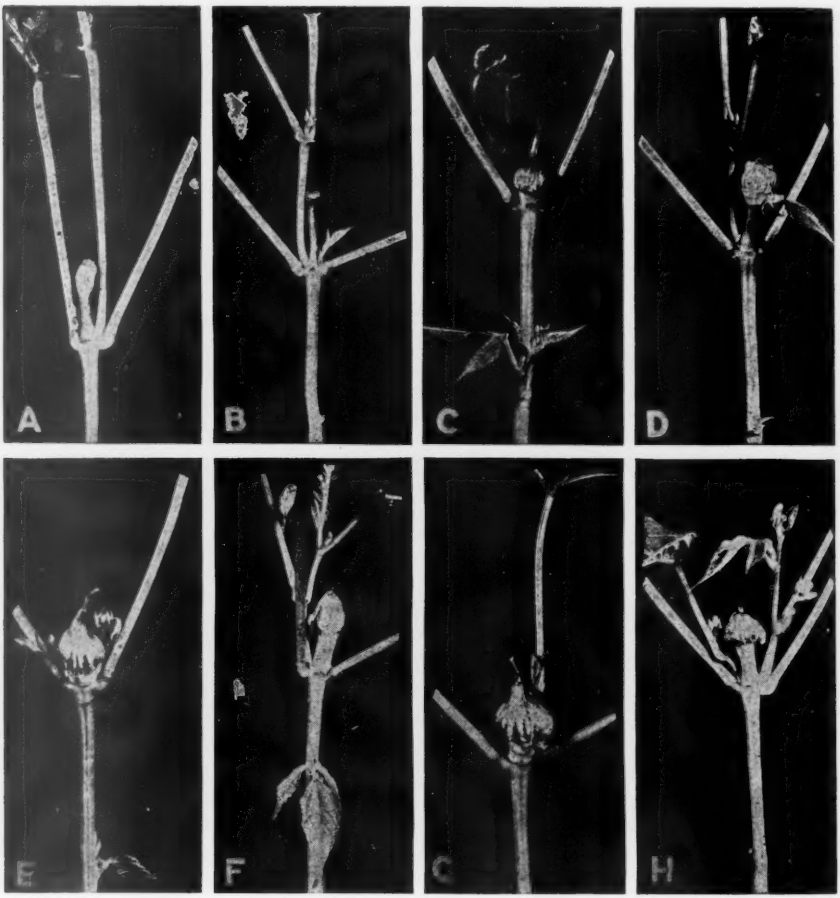

FIG. 1.—Effects of substituted phenoxy compounds on second internode of bean at 16 days following application. *A*, 2-chlorophenoxyacetic acid in Carbowax 1500; *B*, same in lanolin; *C*, 4-chlorophenoxyacetic acid in Carbowax; *D*, same in lanolin; *E*, 2,4-dichlorophenoxyacetic acid in Carbowax; *F*, same in lanolin; *G*, 2,4,5-trichlorophenoxyacetic acid in Carbowax; *H*, same in lanolin. Protruded roots evident in *E* and *G* but in no others.

trichlorophenoxyacetic acid had been applied, the effects were not nearly so pronounced as when the substances had been applied to the bases of the heart-shaped leaves.

Although slight color changes were apparent in the decapitated stems within 24 hours following application of the mixtures, little gross response was noticeable until 30 hours or more had elapsed. A faint yellowing of the tissues first became apparent within 24 hours just below the cut surface to which the mixtures had been applied. By the end of 48 hours the yellowing was more pronounced and the swelling greater. Enlargement continued steadily for several days, the tumors ordinarily reaching their ultimate size in 12–14 days. They then remained relatively constant in size for at least 2 weeks longer. Many, perhaps most, tumors attained a diameter of three to four times that of the basal half of the treated internode, except following application of 2-chlorophenoxyacetic acid; the latter tumors seldom reached a diameter twice that of the lower portion of the second internode (fig. 1*A*, *B*). Although root primordia were commonly developed, only a few of the treated stems ever showed protruded root tips (fig. 1*E*, *G*), and then only following treatment with 2,4-D and 2,4,5-trichlorophenoxyacetic acid in Carbowax.

Histological changes

Definite responses were apparent in sections of the second internode to each growth-regulating substance 24 hours after application, with the least responses following the application of 2-chlorophenoxyacetic acid. This substance applied in lanolin usually induced little response—often none. When Carbowax was the carrier, responses were commonly evident in 24 hours, although some stems responded so little that practically no swelling could be detected, and sections showed little effect even after 72 and 96 hours. Because of the slight responses, no photomicrographs of these stems are included.

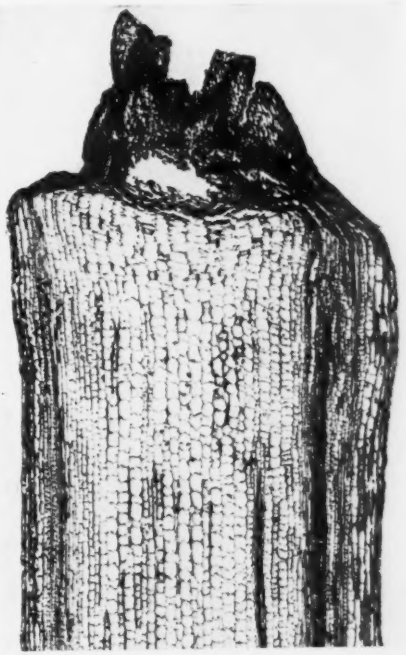

Fig. 2.—Longisection of decapitated second internode 72 hours after apical treatment with Carbowax alone. Many tiers of cells killed and shrunken at and below point of application, with several layers adjacent to these beginning to divide. Tissues at right side show effects of Carbowax which melted and ran over surface of stem.

Responses to applications of 4-chlorophenoxyacetic acid in both Carbowax and lanolin were generally evident at 24 hours, although the responses were much greater to the lanolin mixtures (fig. 3*B*). The cortical cells had elongated radially; their cytoplasmic content had become denser. Many endodermal cells had divided tangentially. Active divisions had

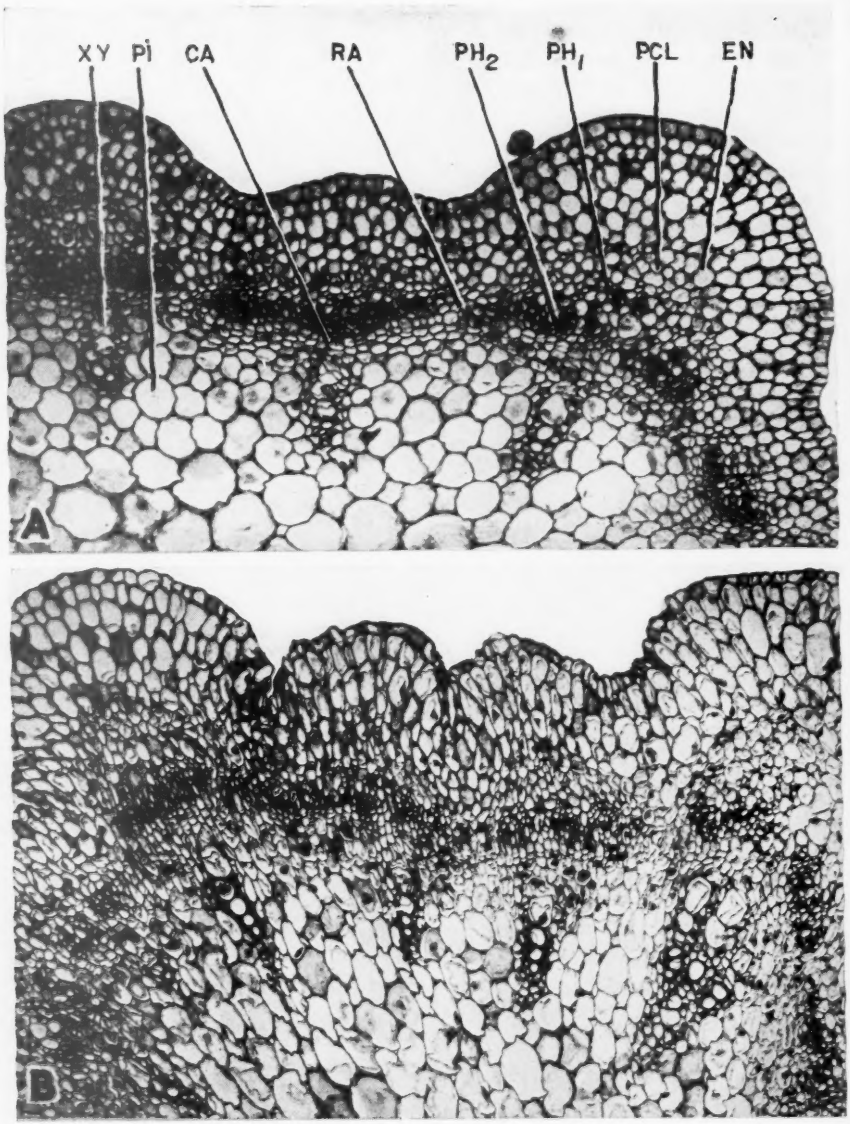

FIG. 3.—*A*, transection near top of second internode of bean at time of treatments, showing various tissues and stage of development (*xy*, xylem; *pi*, pith; *ca*, cambium; *ra*, ray; ph_2, secondary phloem; ph_1, primary phloem; *pcl*, pericycle; *en*, endodermis). *B*, second internode 24 hours after treatment with 4-chlorophenoxy-acetic acid in lanolin; activity beginning in endodermis, cambium, and ray parenchyma, with only slight activity in phloem parenchyma; cortical and pith cells radially elongated.

started in the cambium, phloem, and ray parenchyma cells, but the pericycle appeared unaffected. Although the cells of the pith showed radial elongation, there were no evidences of increased meristematic activity.

Only slight response resulted at 24 hours from applications of 2,4-D in Carbowax. Although the cortical cells showed radial elongation, little effect was evident in endodermis, phloem, cambium, ray parenchyma, or pith (fig. 4*A*). In contrast, application of this compound in lanolin resulted in division of some cells of the endodermis, with considerable activity in the cambium and ray parenchyma and a smaller amount in the phloem. The pith cells showed radial elongation but apparently no increase in the rate of divisions (fig. 4*B*).

At 24 hours essentially no changes could be detected from application of 2,4,5-trichlorophenoxyacetic acid in Carbowax (fig. 5). Applied in lanolin, however, this substance induced marked responses in the cambium and ray parenchyma, with less in the phloem. Cells of the cortex, endodermis, pericycle, and pith, except for radial elongation, were relatively unchanged (fig. 5*B*). The epidermis was unresponsive to all the compounds in these experiments.

At 33 hours the responses were much more evident. Proliferation of endodermis, cambium, phloem, and ray parenchyma had continued, although at a greater rate in some stems (figs. 6*A*, 7*B*) than in others (figs. 6*B*, 7*A*). In general, however, the early responses occurred at a more rapid rate in those stems to which the growth-regulating substances had been applied in lanolin as the carrier.

At 72 hours the responses were similar to all the compounds (figs. 8*A*, *B*, 9*A*, *B*), except 2-chlorophenoxyacetic acid, which commonly induced relatively little response. The total amount of activity had been greater in the internodes to which 2,4-D and 2,4,5-trichlorophenoxyacetic acid were applied, especially when lanolin had been used as the carrier. Definite root primordia were not yet evident.

At 144 hours the stems to which 2,4-D and 2,4,5-trichlorophenoxyacetic acid had been applied in Carbowax showed well-developed adventitious root primordia (figs. 10*A*, 11*A*), but the stems to which the same substance had been applied in lanolin had not developed root primordia (figs. 10*B*, 11*B*), although some suggestions of root primordia showed (fig. 11*B*); 4-chlorophenoxyacetic acid had not stimulated root formation when applied in either carrier. The induced roots originated in the pith rays, where they may occur in one to several of the rays, as reported for their origin in the first internodes in the previous study (1).

Some second internodes to which 4-chlorophenoxyacetic and 2,4-dichlorophenoxyacetic acids were applied in Carbowax showed well-formed roots internal to the cambium at 17 days following application of the mixtures (figs. 12*A*, 13*A*). This development has not been reported heretofore for the bean following treatment with any growth-regulating substance, although it has been described for the tomato (2), *Mirabilis* (3), and *Iresine* (5). Roots external to the cambium were not induced by 4-chlorophenoxyacetic acid applied in Carbowax (fig. 12*A*), nor were well-formed normal-appearing root primordia developed when this substance was applied in lanolin (fig. 12*B*). In spite of the earlier marked proliferation following after the application of all these compounds in lanolin as a carrier, the Carbowax mixtures had induced greater total responses by the sixth day. This advantage was not lost

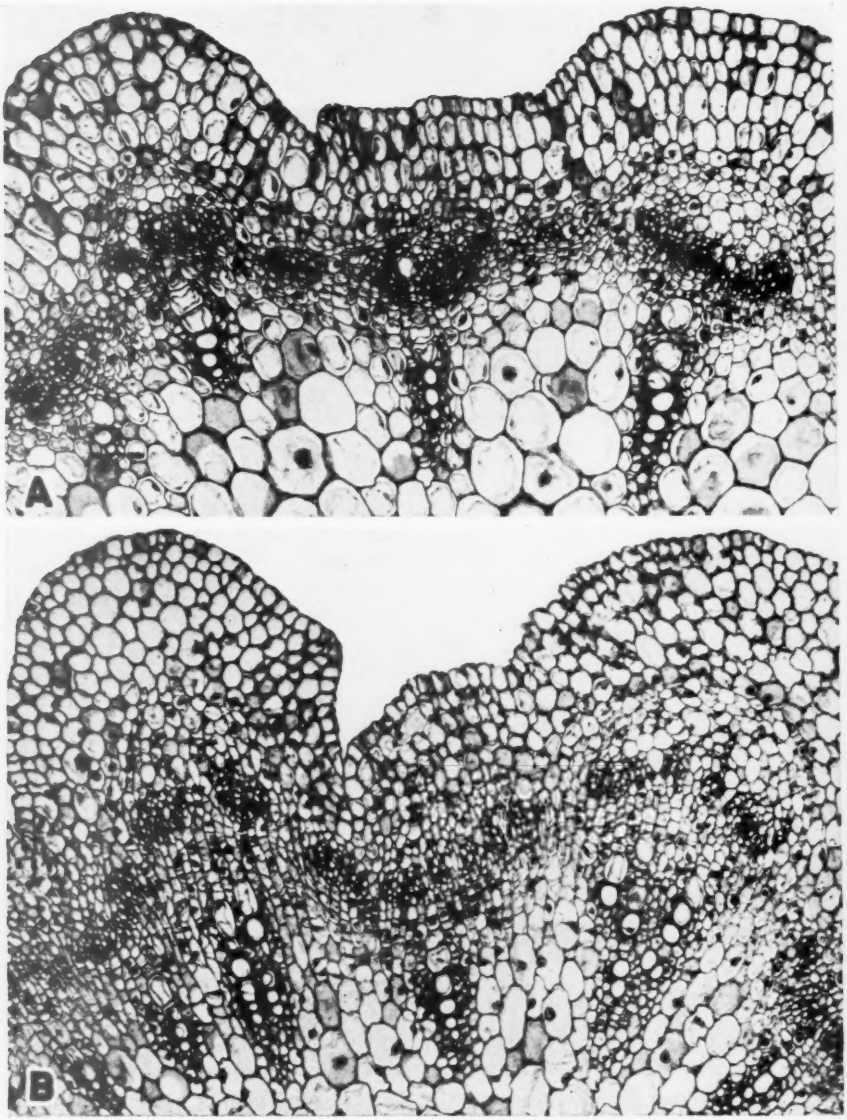

FIG. 4.—Second internode 24 hours after apical treatment with 2,4-dichlorophenoxyacetic acid: *A*, in Carbowax, showing practically no induced activity; and *B*, in lanolin, showing slight activity in endodermis and phloem parenchyma and fairly pronounced activity in cambium and ray parenchyma. *A* magnified slightly more than *B*.

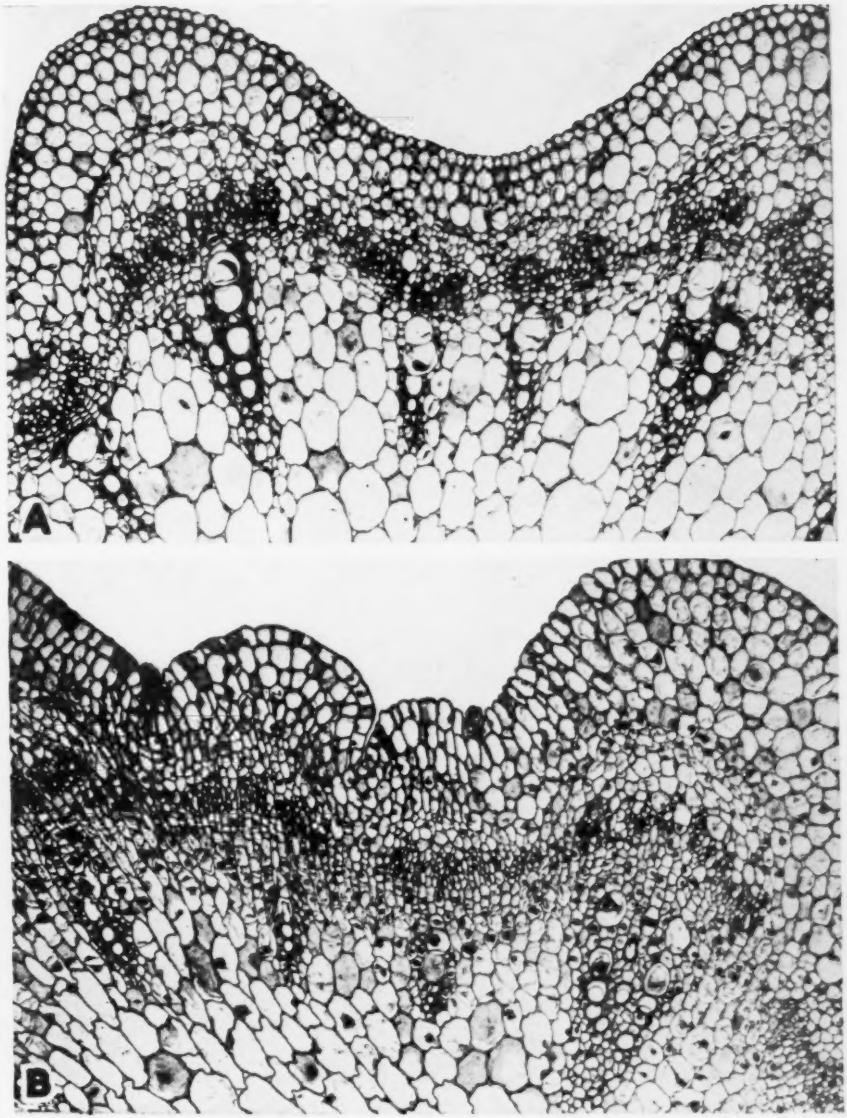

FIG. 5.—Second internode 24 hours after apical treatment with 2,4,5-trichlorophenoxyacetic acid: *A*, in Carbowax, showing little or no induced activity. *B*, in lanolin, showing activity beginning in endodermis and phloem parenchyma and greater responses in cambium and ray parenchyma. Pith and cortex apparently unaffected.

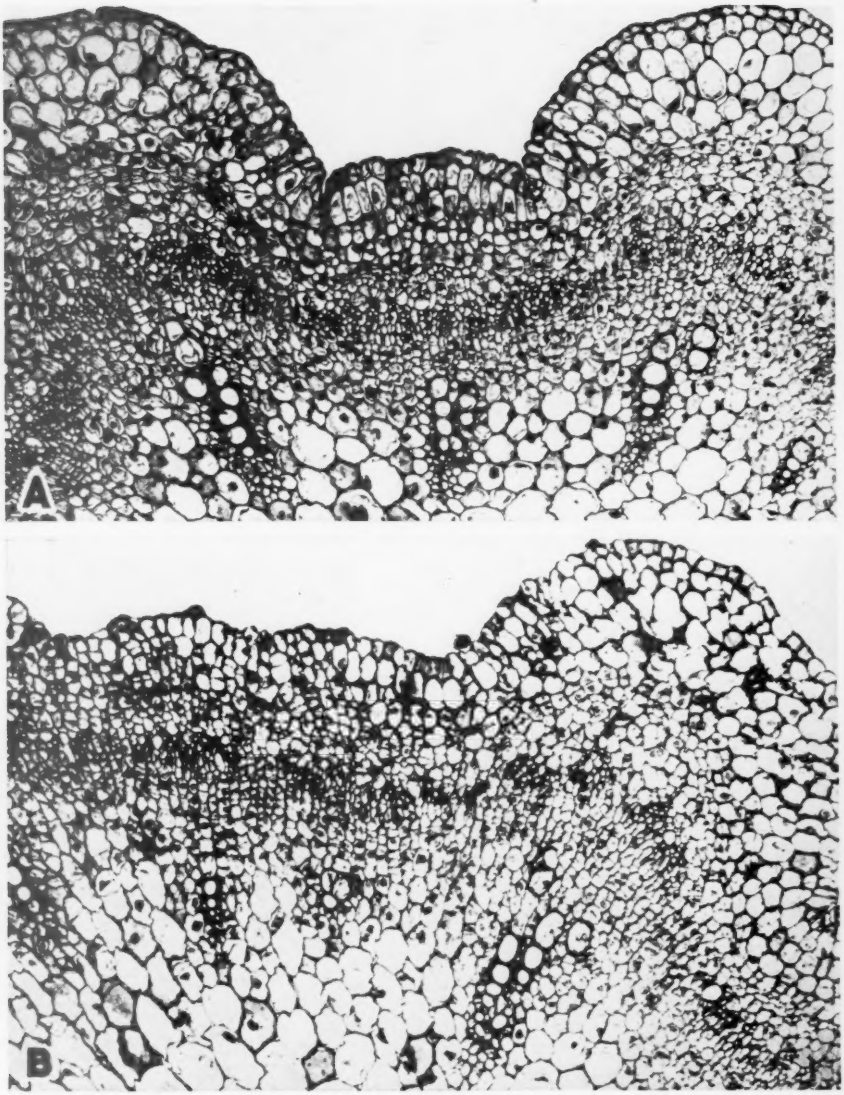

FIG. 6.—Second internode 33 hours after apical treatment: *A*, with 4-chlorophenoxyacetic acid in lanolin, showing responses in endodermis, cambium, and ray parenchyma; little response in phloem. *B*, 2,4,5-trichlorophenoxyacetic acid in lanolin. Same tissues have responded as in *A*.

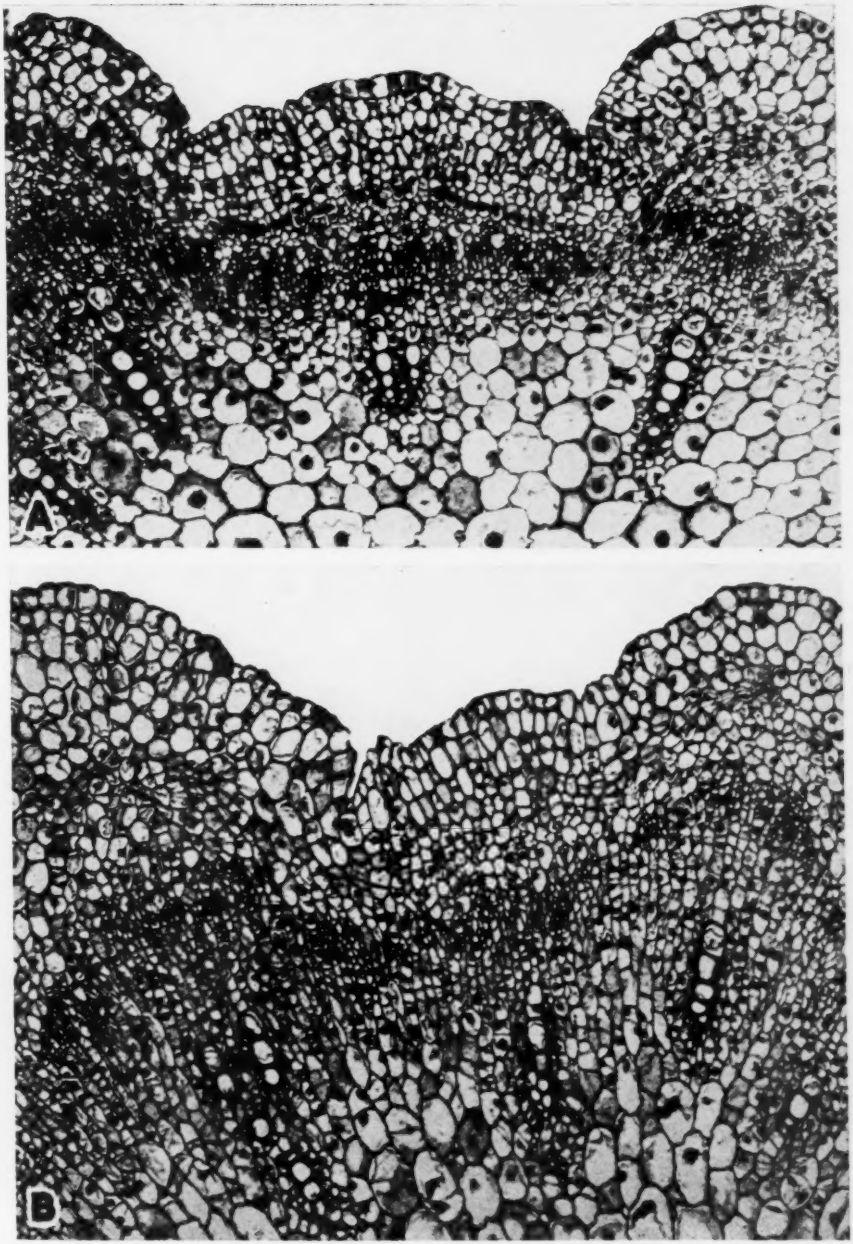

FIG. 7.—Second internode 33 hours after apical treatment with 2,4-dichlorophenoxyacetic acid: *A*, in Carbowax, showing only slight responses in endodermis, cambium, phloem, and ray parenchyma. *B*, in lanolin, showing greater activity in all these tissues, especially in cambium and ray parenchyma.

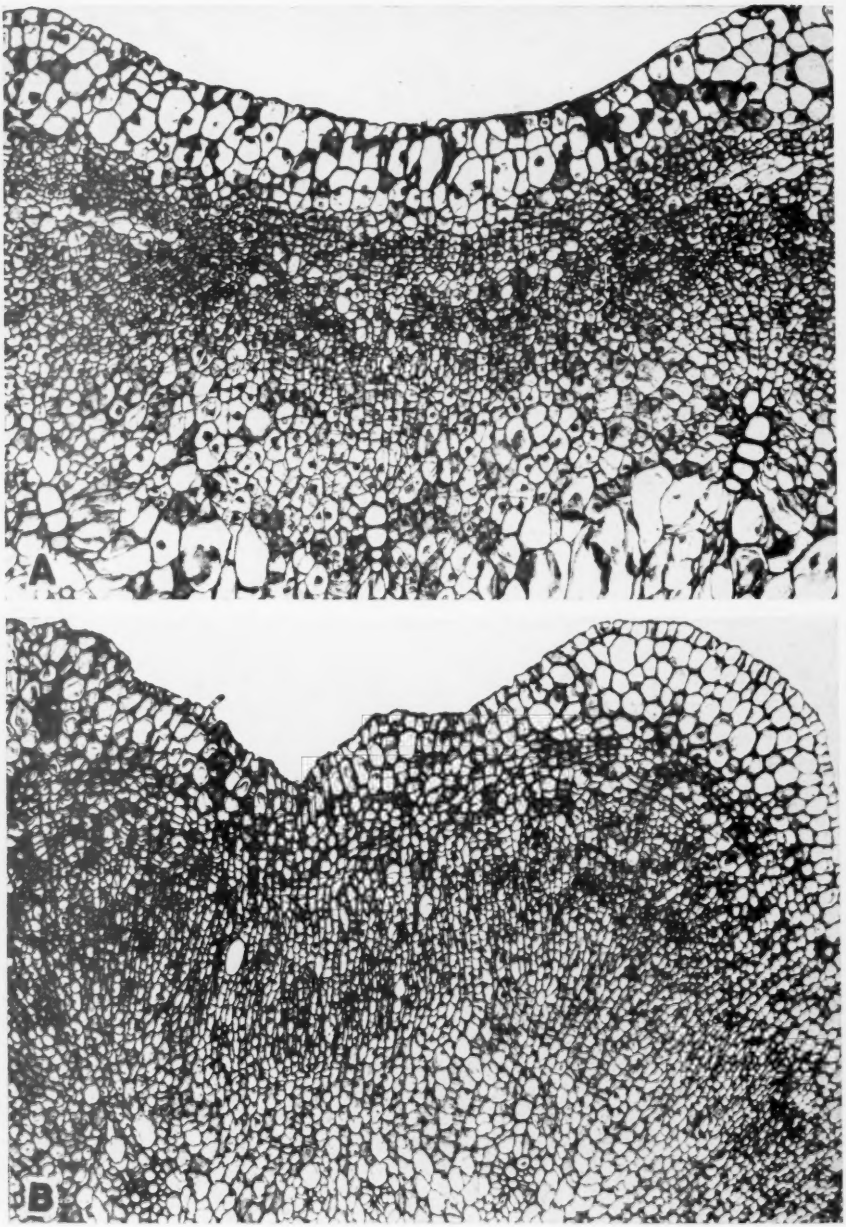

FIG. 8.—Second internode 72 hours after apical treatment: *A*, with 4-chlorophenoxyacetic acid, showing activity in endodermis, cambium, phloem, and ray parenchyma; outer cortical and some pith cells enlarged. *B*, with 2,4,5-trichlorophenoxyacetic acid in lanolin, showing marked proliferation of endodermis, cambium, phloem, and ray parenchyma. Outer pith cells and a few inner cortical cells have been responsive.

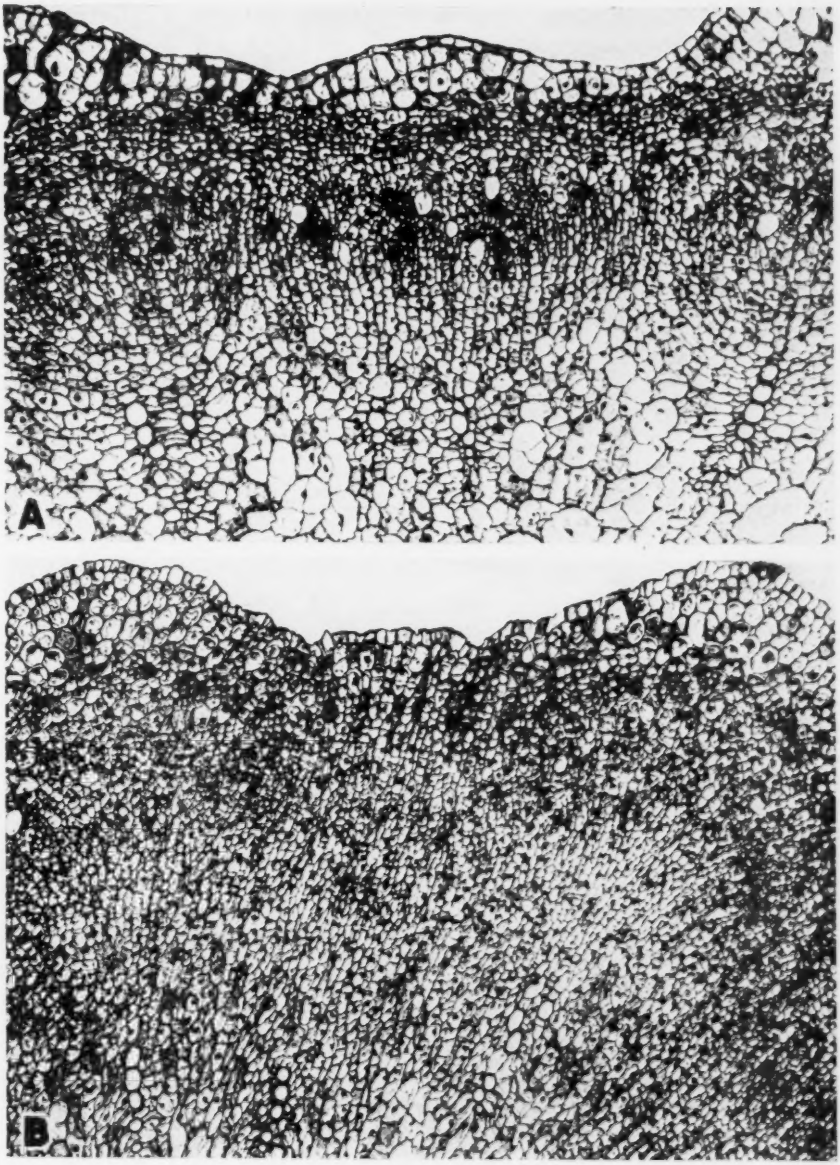

FIG. 9.—Second internode 72 hours after apical treatment with 2,4-dichlorophenoxyacetic acid: *A*, in Carbowax. *B*, in lanolin. Same tissues respond in both stems, but rates and degree of response are different. Cambium, ray, and phloem parenchyma much more active in *B*, although endodermis has responded essentially the same in both. Inner cortex slightly active in both.

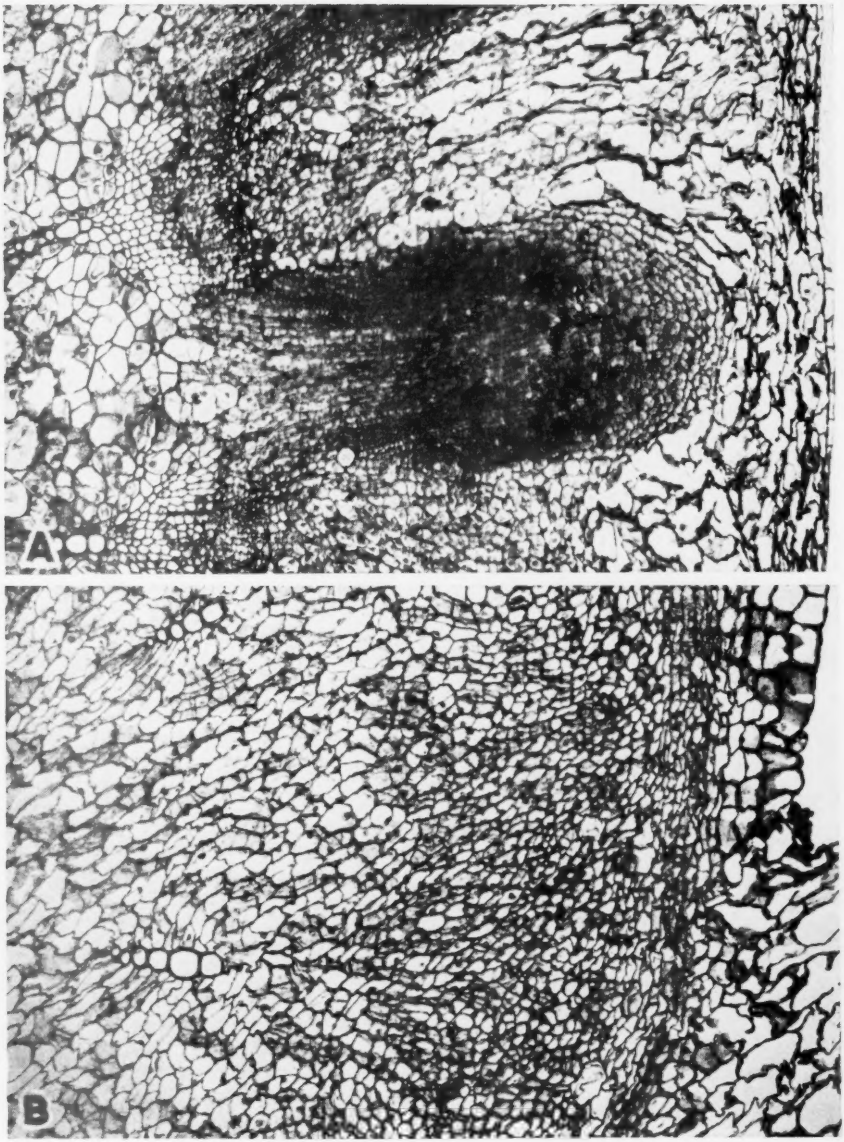

FIG. 10.—Second internode 144 hours after apical treatment with 2,4-dichlorophenoxyacetic acid: *A*, in Carbowax. *B*, in lanolin. Stems treated with Carbowax mixture now show much greater responses, with well-developed root primordia in pith rays, while those treated with lanolin mixture have responded relatively little more than at 72 hours. (Cf. figs. 9*B* and 10*B*.)

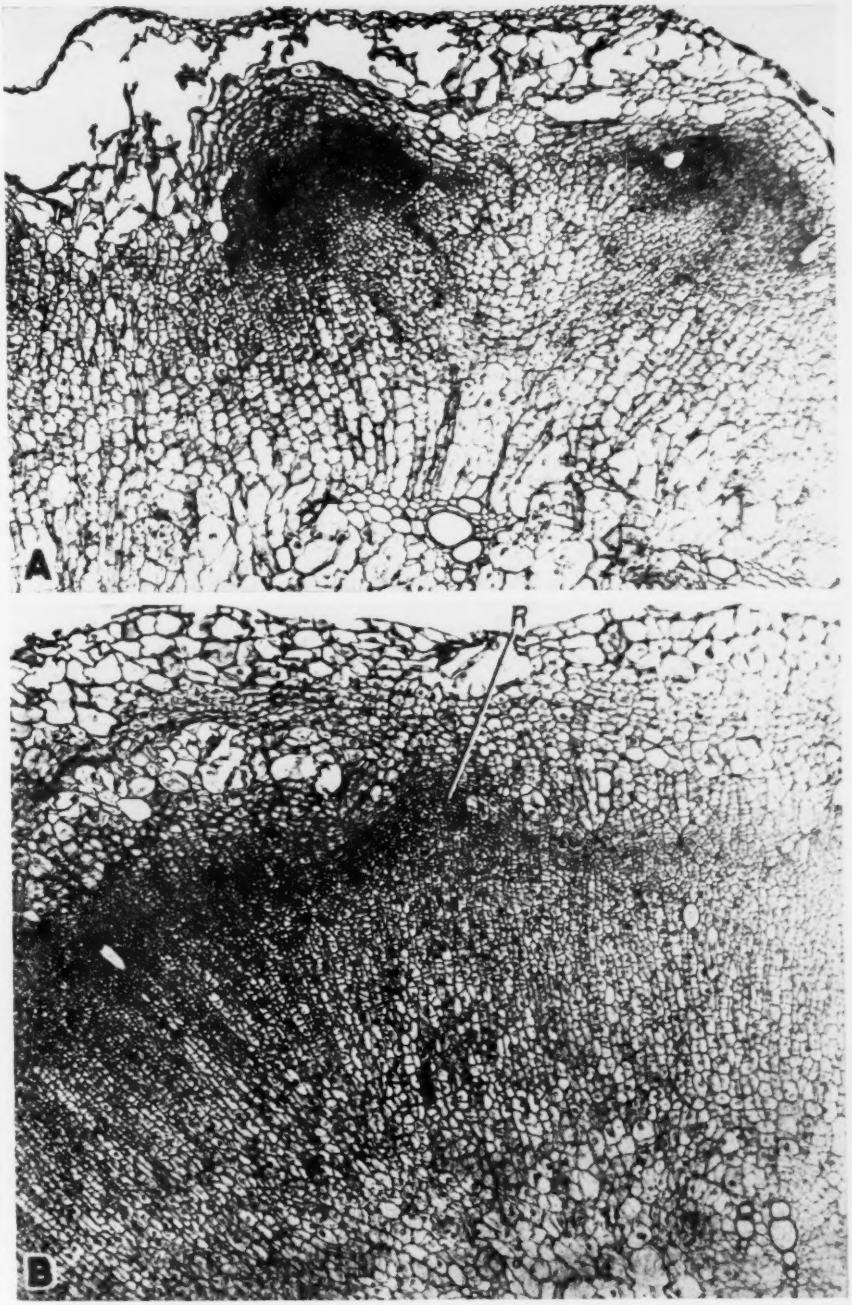

FIG. 11.—Second internode 144 hours after apical treatment with 2,4,5-trichlorophenoxyacetic acid: *A*, in Carbowax. *B*, in lanolin. Tissues involved and their responses similar to those in fig. 10*A* and *B*, respectively. What appears to be a developing root primordium at top center of *B* does not develop into a well-organized root (see fig. 14*B*). Endodermis and cortex chiefly crushed and dead in figs. 10*A* and 11*A*.

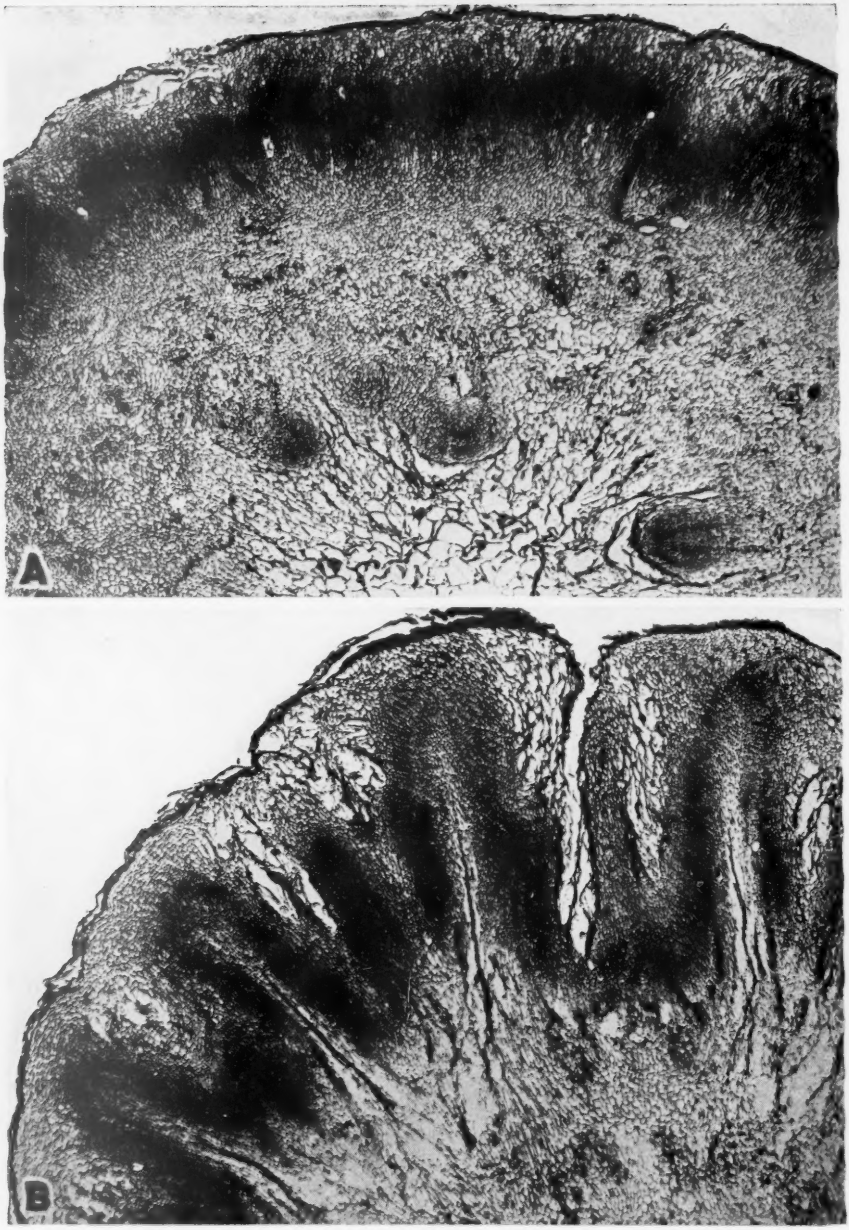

FIG. 12.—Second internode 17 days after apical treatment with 4-chlorophenoxyacetic acid: *A*, in Carbowax, marked activity throughout most of stem; a few adventitious roots developed in pith, but no well-organized roots developed external to cambium. *B*, in lanolin, showing neither internal roots nor well-organized external ones; although considerable proliferation occurs in endodermis, phloem, cambium, and ray parenchyma, well-developed root primordia were never organized. Death of cells in some tissues has begun.

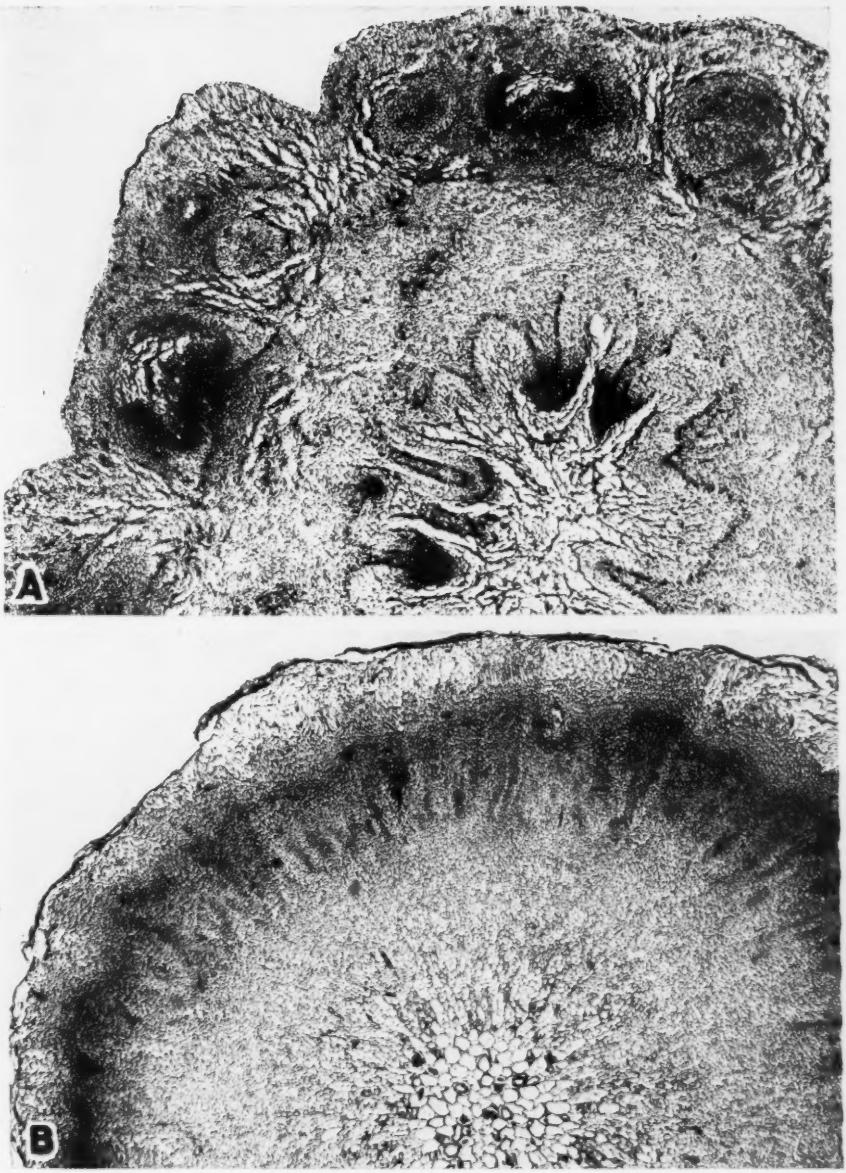

FIG. 13.—Second internode 17 days after apical treatment with 2,4-dichlorophenoxyacetic acid: *A*, in Carbowax, showing well-organized internal adventitious roots and less well-organized ones external to cambium. *B*, in lanolin, showing marked proliferation throughout, except in central pith, pericycle, outer cortex, and epidermis, but with no adventitious roots developed.

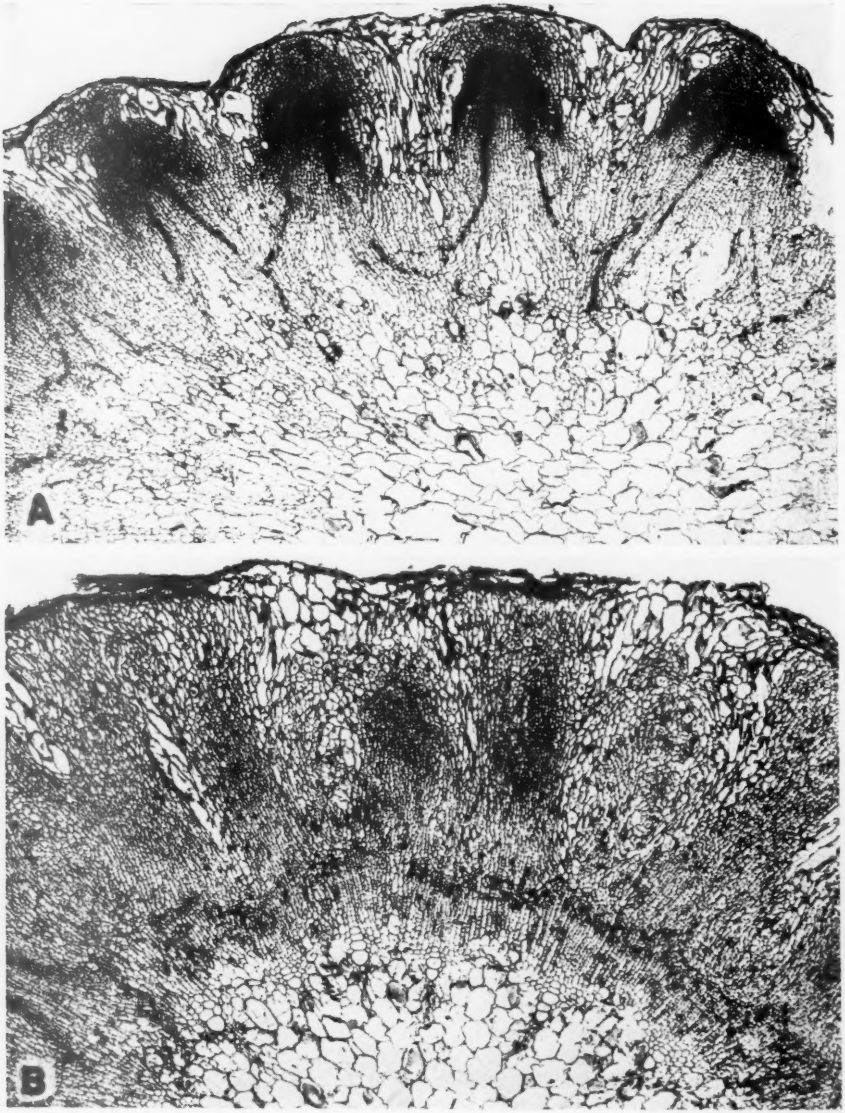

FIG. 14.—Second internode 17 days after apical treatment with 2,4,5-trichlorophenoxyacetic acid: *A*, in Carbowax, showing well-organized adventitious root in each ray. *B*, in lanolin, showing considerable proliferation in ray and phloem parenchyma and smaller amount in cambium but with no definitely organized roots present.

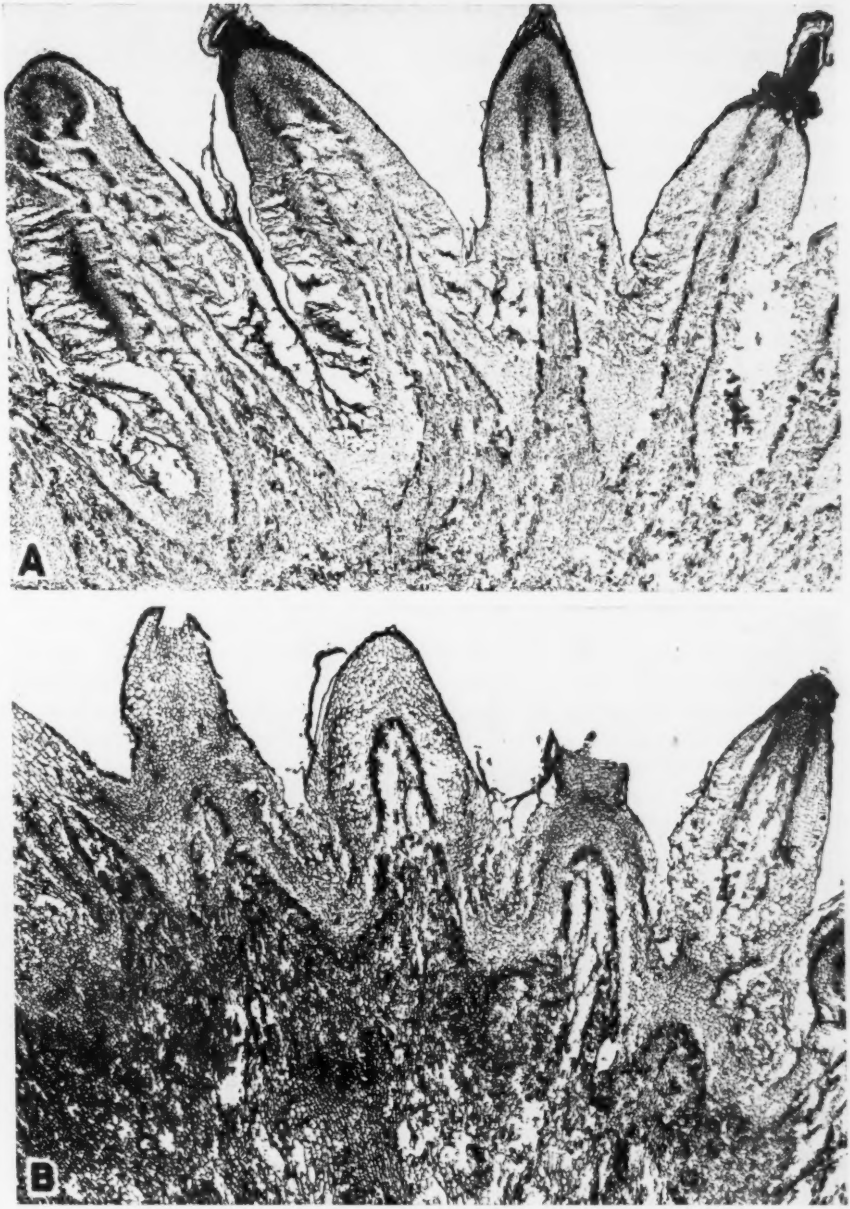

FIG. 15.—Second internode 26 days after apical treatment with 2,4-dichlorophenoxyacetic acid in Carbowax: *A*, at 1%. *B*, at 0.5%. Responses in general are nearly identical; protruded roots are similar, and numerous dead and disorganized areas are evident throughout much of both sections, including disorganized areas in roots also.

thereafter. All the internodes which showed roots protruded from the tumors had had the phenoxy compounds applied to them in Carbowax (figs. 13*A*, 14*A*, 15*A*, B). Although considerable proliferation of various tissues had occurred in the stems of the plants to which the compounds had been applied in lanolin, well-organized roots were usually not induced, and none was ever extruded through the surface of the galls in these experiments (figs. 12*B*, 13*B*, 14*B*).

About 3 weeks after treatment the induced apical tumors began to dry and die, showing slight shrinkage and wrinkling. There were no observable differences between those resulting from 0.5% and 1% mixtures of the substances employed. Transections of tumors at 26 days following application of 1% and 0.5% 2,4-D in Carbowax (fig. 15*A*, *B*) revealed that almost identical responses had resulted and that both tumors were now beginning to die. Epidermis and cortex were completely dead and crushed, the core and outer portions of induced roots showed dead and disintegrating tissues, and there were numerous localized areas in the phloem, rays, xylem, and pith showing dead and disorganized cells. In a few days the tumors became dry and spongy, but none of the plants of these experiments was killed as a result of the application of any of these phenoxy compounds, as had so generally resulted when 2,4-D and 2,4,5-trichlorophenoxyacetid acid had been applied to the bases of the heart-shaped leaves (1).

Discussion

Responses to certain of the substituted phenoxy compounds in both Carbowax and lanolin when applied to decapitated second internodes of the bean were rather strikingly different from those which resulted when the same mixtures were applied to the bases of the heart-shaped leaves (1). Telemorphic effects were practically absent in the decapitated plants. Generally neither stem curvature nor epinasty was shown by these plants, and the responses were chiefly limited to the upper 10–12 mm. of the treated internodes and to the axillary shoots and their leaves, except in those plants on which the Carbowax mixtures melted and ran down along the surface of the first internode and hypocotyl. Even in these cases the responses were also less than in the plants to which these substances had been applied to the heart-shaped leaves. Although the decapitated plants were older at the time of treatment, and hence not so highly meristematic, this of itself scarcely seems sufficient to account for all the differences in responses. A factor of equal, or perhaps of even greater, significance may be associated with the translocation of organic food materials from active photosynthetic regions, as indicated by the studies of MITCHELL and BROWN (7). Little photosynthesis would occur in the decapitated second internode, and there would be comparatively little basipetal movement. Although this internode had comparatively few mature cells in it at the time of decapitation and treatment, the responses were nevertheless generally restricted to the region near the distal end, except in those instances when the Carbowax mixtures melted and ran down the stems. In most of the latter cases the responses were greater, especially in the first internode and the hypocotyl, indicating that there may have been an association between the movement of the growth-regulating substance and the translocation of organic food materials from the heart-shaped leaves attached at the distal end of the first internode. Light and temperature were apparently not differential factors

of significance in these experiments, since all plants of any set of experiments were grown under as nearly identical environmental conditions as could be managed.

Summary

1. Decapitated second internodes of the Red Kidney bean were treated with four of the substituted phenoxy compounds at 0.5% and 1% concentrations, using both Carbowax 1500 and lanolin as carriers. The gross responses were limited largely to the region at, and immediately below, the place of application of the mixtures and to the axillary shoots and their leaves, except when the Carbowax mixtures melted and ran down the plant axis, often over portions of the first internode and sometimes the upper portion of the hypocotyl. Swelling and often root development occurred under these areas. Development of axillary shoots was generally retarded, and frequently their leaves were dwarfed, mottled, or otherwise deformed except following 2,4,5-trichlorophenoxyacetic acid. Application of 2-chlorophenoxyacetic acid induced less response than any of the others. None of the compounds produced epinasty or stem curvature in these experiments, and none of the plants was killed as a consequence of the treatment.

2. Studies of the histological responses were confined to the second internode. The early responses were most evident when lanolin was the carrier, but at 6 days and subsequently the Carbowax mixtures induced greater responses.

3. Responses occurred chiefly in the endodermis, cambium, phloem, and ray parenchyma. The inner cortical cells and the pith were responsive in some stems, especially following application of 2,4-dichlorophenoxyacetic acid in Carbowax. Epidermis, outer cortex, and pericycle were generally unresponsive to all the mixtures.

4. Although some roots may develop internal to the cambium following treatment with 4-chloro- and 2,4-dichlorophenoxyacetic acids, no well-formed roots external to the cambium were found following application of 2- or of 4-chlorophenoxyacetic acids on any plants. Well-formed protruded roots were found only following treatment with 2,4-di- and 2,4,5-trichlorophenoxyacetic acids in Carbowax.

5. Differences in responses between the plants in this experiment and those in which the growth-regulators were applied to the bases of the heart-shaped leaves may in part be associated with greater age of the decapitated plants when treated. A factor of much greater possible significance, however, may be the association of transport of the growth-regulating substances with the movement of organic foods. In the decapitated plants little movement of organic foods occurs in the decapitated second internode and little movement of the growth substance. This may possibly account for the limited telemorphic effects in the decapitated plants as compared with the pronounced responses when the same substituted phenoxy compounds were applied to the bases of the heart-shaped leaves.

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REMOVAL OF 2,4-DICHLOROPHENOXYACETIC ACID AND ITS CALCIUM SALT FROM SIX DIFFERENT SOILS BY LEACHING[1]

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 578

ROBERT W. HANKS

Introduction

Most reports to date on the use of 2,4-dichlorophenoxyacetic acid (2,4-D) as a herbicide deal with spray application to vegetation. The selective nature of the substance has been well demonstrated. The direct treatment of soils with 2,4-D in order to kill weed seeds and for other purposes is chiefly in the experimental stage. Published results of such experiments suggest that the length of persistence of the toxic effect is affected by soil type and management practice. There seems to be considerable variation in persistence of 2,4-D in soils: from less than 4 weeks in muck (**3**) to as much as 18 months in soil stored in a relatively dry condition (**5**). The response of clover indicated a difference in the persistence of the acid in Rothamsted clay and Woburn sand (**6**). KRIES (**4**) found a longer period of toxicity in soil to which lime had been added a few days prior to application of the acid than in unlimed soil; she noted, however, that additions of partially decomposed leaf mold at the time of liming tended to reduce the degree and persistence of toxicity in the limed soil. BALL (**1**) reported that toxic effects of the acid were more pronounced in one of three soils tested than in the others, and for a longer period of time. 2,4-Dichlorophenoxyacetic acid was more readily leached from soil than were other herbicides (**2**), although it is still unknown whether the disappearance of the toxic effect is due chiefly to leaching, to biological decomposition, to a chemical or a physical combination with components of the soil, or to all these causes.

The purpose of the experiment here reported was to determine the relative rates at which 2,4-dichlorophenoxyacetic acid and its calcium salt could be leached from soils possessing different physical and chemical properties. The calcium salt was used in order to determine if it would be more persistent or less readily leached than the acid; this possibility was indicated by KRIES's results (**4**) on limed soils. Acid and alkaline soils were tested in order to investigate further a possible relationship between soil reaction and persistence or degree of toxicity of the herbicides.

[1] This work was aided in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

Methods

The sources and some properties of the six soils which were tested are listed in table 1. Soil pH was determined with the glass electrode, moisture equivalent by the centrifuge method.

Each soil lot was thoroughly air-dried and passed through a $\frac{1}{4}$-inch screen in order to obtain a uniform maximum size of soil particles. A small aliquot of each soil was then removed to serve as a control, receiving no chemical treatment. With the exception of soil F, half of each soil was treated with lime ($CaCO_3$) at a rate approximately equivalent to 2000 pounds per acre to a 6-inch depth. Because it was naturally alkaline, only a small aliquot of soil F was limed. The limed aliquots were placed in flats in the greenhouse and were kept moist (not leached) for 2 weeks to permit the reaction of the calcium with other constituents of the soil to approach equilibrium.

In order to obtain a uniform distribution of 2,4-D and its calcium salt within the soils, sand-herbicide mixtures were prepared quantitatively by pouring an ethyl alcohol solution of the acid and an ethyl alcohol mixture of the salt over pure quartz sand. It was necessary to use the alcoholic mixture of the calcium salt rather than a solution because the salt is insoluble in water, ethyl alcohol, diethyl ether, petroleum ether, acetone, or chloroform. Each batch of treated sand was air-dried and mixed to insure uniformity of this material used in treating the soils.

The sand-acid mixture and the sand-salt mixture were applied to the soils as follows: half of the limed and half of the nonlimed aliquots of each soil were treated with the sand-acid mixture to supply 20 p.p.m. by weight of the acid in the soil; the remaining aliquots of limed and nonlimed soils were treated with the sand-salt mixture to give approximately 22.3 p.p.m. by weight of the calcium salt in the soil. Thus there was applied in equal weights that part of the molecule of these herbicides which is considered to be responsible for their toxic effects (8). Thus, four conditions were obtained for each soil type: (1) lime with the acid, (2) lime with the calcium salt, (3) the acid without lime, and (4) the calcium salt without lime.

After incorporating the sand-herbicide mixtures with the soils, 4-inch glazed crocks with side-hole drains were filled to a depth of approximately 6 inches. Each drain had been covered with glass wool and fitted with a cork stopper and glass tube to facilitate collection of the leachate in Erlenmeyer flasks. There was a minimum of three pots of soil for each treatment of soil A; five or six pots were available for each treatment of the other five soil types.

TABLE 1

Soil	Source	Physical characterization	pH	Moisture equivalent
A	Beltsville, Md.	Sandy	3.8	10.99
B	Lake Geneva, Wis.	Silt loam	7.7	15.57
C	Lake Geneva, Wis.	Peat	6.7	135.04
D	Chicago, Ill.	Clay loam	8.4	28.55
E	Gary, Ind.	Sandy loam	7.1	15.23
F	Hurley, N.M.	Sandy clay	8.4	25.50

The potted soils were placed in a basement laboratory in order that water loss by evaporation might be reduced to a minimum and to provide uniform temperature conditions. All pots were wetted initially, with the drains closed, with the equivalent of 4 surface-inches of water. This permitted each soil mass to become thoroughly wetted before any leachate was collected. In addition to the initial 4 surface-inches of water, one-half of the pots in each soil treatment received the equivalent of 10 surface-inches of water over a 6-week period; the other half received the equivalent of 16 surface-inches in the same period. The 10 surface-inches of water were applied at the rate of 1 inch per week for the first 2 weeks and 2 inches per week thereafter. The 16 surface-inches were added at the rate of 2 inches per week for the first 2 weeks and 3 inches per week thereafter. The temperature in the laboratory was 26° C. throughout the experiment.

The leachate from each soil treatment was tested once a week for the presence of herbicide by a slight modification of the bio-assay developed by SWANSON (7). In his method SWANSON used hybrid field corn of the Silver King (Wisconsin no. 7) variety germinated for 48 hours in 6-inch Petri dishes. Twelve to fifteen seedlings with roots between 15 and 25 mm. in length were then placed on filter paper in other 6-inch Petri dishes and moistened with 15 ml. of solution containing 2,4-dichlorophenoxyacetic acid. After 48 hours the increase in average root length of the seedlings in each dish was determined and expressed as a percentage of the increase in average root length of control seedlings treated with distilled water. All germination and growth were in darkness at 26° C. SWANSON determined concentrations of the acid ranging from 1/100 p.p.m. to 2 p.p.m. with only a 5% error.

The bio-assay used in the writer's experiment measured the relative toxicity of the leachates rather than the absolute concentration of the herbicide for which the test was devised originally. SWANSON's method was modified by use of 9-cm. Petri dishes, ten seedlings to the dish treated with 10 ml. of leachate. All other conditions of the original method were unchanged.

Results and discussion

The results of the weekly bio-assays on leachates from soil samples leached with the equivalent of 10 and 16 surface-inches of water showed similar trends within each soil type. Therefore, only the results obtained from the application of the larger amount are reported graphically (fig. 1). The increase in average root length of seedlings grown in leachate from each soil treatment is expressed as a percentage of the increase in root length of seedlings grown in the leachate from untreated control soil of the same type. Each bar of the graph shows this percentage value obtained from the bio-assay of a single leachate. A block of six bars summarizes the results from a single soil treatment, each bar of a block, from left to right, representing the results of each successive week of the 6-week period. Percentage values much greater than 100 may be considered to indicate a stimulation in growth rate caused by the effects of the herbicide; those much below 100 show the relative degree of toxicity of the various leachates.

The results of the bio-assays indicate no significant or consistent difference in the degree of toxicity, in comparable leachates, of the acid and of the calcium salt at the concentrations used. The cal-

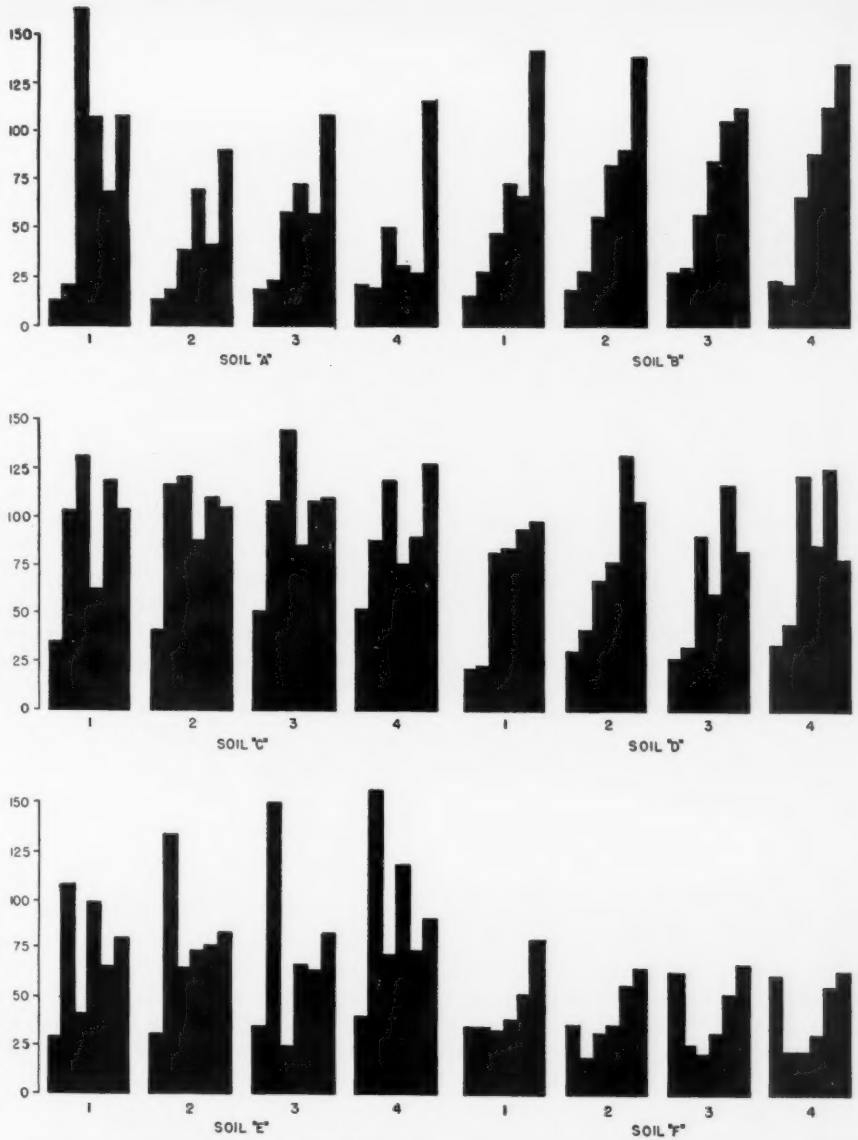

FIG. 1.—Percentage of corn-root elongation in leachate from each soil treatment based on its own control, indicating extent and rate of loss of herbicidal effect. Each bar of a block gives percentage value from bioassay on single leachate. A block of six bars summarizes results from a single soil treatment, each bar from left to right representing results of each successive week of the 6-week period. Numbers below each block designate soil treatments: *1*, limed soil with 2,4-dichlorophenoxyacetic acid; *2*, limed soil with calcium salt of 2,4-D; *3*, nonlimed soil with 2,4-D; *4*, nonlimed soil with calcium salt.

cium salt, or its reaction products, thus seems to be as soluble as the acid in the soil solution of the tested soils, although the calcium salt is insoluble in water, ethyl alcohol, diethyl ether, petroleum ether, acetone, or chloroform. In general, the addition of lime to the soils likewise seemed to produce no significant differences in toxicity of leachates. In contrast, KRIES (4) found a prolongation of toxic effects of 2,4-D in soil in the presence of added lime, although the soil she used was different from those tested by the writer. It may be, also, that the nature of the leachate from soil is such that the degree of sensitivity in the bio-assay is reduced. However, the toxic effects of the herbicides persisted longer in one of the naturally alkaline soils (F) than in any of the other soils tested.

The bio-assay indicated that the leachate from soil C (peat) was relatively free of toxicity at the end of 2 weeks. HAMNER *et al.* (3) reported that the toxic effects of 2,4-D in muck soil disappeared in less than 4 weeks; KRIES (4) found that organic matter added to soil treated with both lime and 2,4-D reduced the period of toxicity. Thus it seems that organic matter in some way reduces the period of toxicity of these herbicides in soil.

The cause of the great fluctuation in the results obtained during the first 3 weeks with soil E is not known. With the fluctuation present in all four treatments of this soil, some unknown, and therefore uncontrolled, factor may have been operating in the control samples of that soil.

The relative toxicity in the successive leachates from soil B decreased at a more uniform rate than in the leachates from the other soils. Soil C was apparently rendered relatively free of toxicity with as little as 4 surface-inches of water applied over a 2-week period, whereas toxic effects were still present in the leachate from soil F after treatment with four times as much water over the 6-week period. Besides leaching, other factors, such as biological decomposition, adsorption by colloidal systems in the soil, and chemical reactions forming nontoxic compounds may be involved in reducing toxicity.

At the end of 6 weeks of leaching treatment, all pots were moved to the greenhouse, and the soil was allowed to dry for 5 days. The soil from each pot was then mixed in such a manner that, when returned to the original pot, the portion that had been at the bottom was uppermost. Thus, any herbicide which had not been removed by leaching from the bottom layers of soil was immediately in contact with germinating seeds. Each pot was planted with ten red kidney beans to determine any residual effects of the herbicide.

This test indicated that soil F was the only soil from which all toxic effects due to the herbicide had not yet disappeared, thus confirming the results of the bio-assay. In this soil, the toxic effects were found to be greater in those pots which had been leached with only 10 surface-inches of water than in those leached with 16 inches. The toxicity did not affect the percentage of germination and was shown chiefly in the malformation and mottled appearance of the primary and first trifoliate leaves.

Summary

1. Six soils with a wide range in pH and in physical properties were treated with lime, 2,4-dichlorophenoxyacetic acid, and the calcium salt of the acid in such a manner as to obtain four conditions for each soil.

2. The treated soils were leached with

water at rates equivalent to 10 and 16 surface-inches over a 6-week period. The leachates were collected and tested weekly with a corn-seed bio-assay to determine relative toxicities.

3. The bio-assays indicated that the leachates from soil C (peat) were relatively nontoxic at the end of 2 weeks and that those from all soils except soil F (naturally alkaline) were relatively free of the herbicides at the end of 6 weeks. There were no significant differences in the degree of toxicity and its persistence in limed as compared with unlimed samples within each soil type or in that of the calcium salt versus the acid.

4. At the end of 6 weeks, the bottom half of each soil mass was tested for residual toxic effects by the germination of red kidney beans. This test indicated that soil F was still somewhat toxic from the treatment and that the samples of this soil leached with 10 surface-inches of water were more toxic than those leached with 16 surface-inches of water. All other soils were then free of the toxic effect of the herbicide.

The author wishes to express gratitude to Dr. CHARLES E. OLMSTED for advice and close interest in the problem.

DEPARTMENT OF BOTANY
UNIVERSITY OF MIAMI
CORAL GABLES, FLORIDA

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HISTOLOGICAL RESPONSES OF BEAN PLANTS TO NICOTINE AND TO WOUNDING

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 579

A. GERALDINE WHITING AND MARY AILEEN MURRAY

Introduction

Nicotine is one of the well-known plant alkaloids, a group of compounds which have been used in many different types of experiments. Some of these alkaloids possess medicinal properties, and many are intensely poisonous, their effect depending in part upon the concentration used. According to MACHT and DAVIS (12), nicotine is very toxic not only for animal but also for living plant protoplasm. They recorded inhibition of root growth of *Lupinus albus* in 1:5,000 solutions of various forms of nicotine. Likewise, when EISLER and PORTHEIM (5) soaked certain seeds in 0.1–0.4% nicotine solutions, tobacco failed to germinate and buckwheat seedlings were injured. In contrast, SONNENSCHEIN (20) found that growth was stimulated when soybean seeds were soaked in a 0.2% solution for 24 hours. He also noted that nicotine in combination with atropine or with colchicine induced polyploidy. NEMEC (17) described certain effects of nicotine upon cell division in root tips of the pea. From experiments with young corn plants grown in nutrient solutions containing nicotine tartrate ($m/100$ to $m/10{,}000$), WEIS *et al.* (22) reported that nicotine was harmless to the plants.

The application of nicotine sulphate as an insecticide has been a widespread horticultural practice for nearly two centuries. In 1940 MARTIN (13) stated that no record could be found of any damage to plants caused by the nicotine itself in washes and dusts used as insecticides. During greenhouse fumigation experiments with concentrations of nicotine as high as 30–60%, RICHARDSON *et al.* (18) observed no apparent injury to crops of lettuce, tomato, and various other plants. More recently WITHROW and BIEBEL (24) reported that young leaves of Biloxi soybean showed chlorosis after exposure to a 14% nicotine fumigant. However, they suggested that other products in the fumigation process may have been involved.

Experimentation with nicotine as a growth-regulating substance was reported by TRAUB (21) in 1938. He tested over one hundred different compounds for their effectiveness in promoting the initiation of roots in cuttings of several different subtropical plants. In comparison with other substances tested, nicotine in a 0.01% solution was rated in the highest degree of superiority. This placed it in the same category as 0.02% indolebutyric acid, which TRAUB considered the most useful of the compounds tested.

Material and methods

The present report on nicotine is a continuation of the series of experiments (3, 7, 8, 9, 10, 15, 23) on the histological response of bean plants (*Phaseolus vulgaris* var. Red Kidney) to various growth regulating substances. Methods were similar to those described for the earlier experiments. Observations and collections of material were made at various intervals up to 30 days after decapitation. The specimens were preserved in Nava-

shin's solution, imbedded by the butyl alcohol–paraffin method, sectioned at 10–15 μ, and stained in a modified triple stain. The nicotine was obtained from the Eastman Kodak Company.

Four different lots of bean plants were used. Because histological examination of the first lot showed marked similarity between treated and control plants, the original experiment was repeated and extended with greater consideration given to control plants and natural wound response.

LOT I.—These plants were grown in the greenhouses of the Bureau of Plant Industry, Soils and Agricultural Engineering, United States Department of Agriculture, Beltsville, Maryland, in May and June, 1939. Young plants of uniform size with the first trifoliate leaf unfolding were decapitated near the apex of the lengthening second internode. The treated plants, the greater part of the lot, received an application of 2% nicotine in anhydrous lanolin smeared over the cut surface. Some of the control plants were only decapitated; on others, lanolin alone was applied to the cut surface.

LOT II.—This experiment, in general a repetition of the first, was conducted in the greenhouses of the University of Chicago in May and June, 1946. The number of control plants equaled that of plants treated with nicotine. One half of the latter received an application of 2% nicotine in lanolin; the other half, 3% nicotine in lanolin. Control plants were treated in two ways: simple decapitation and decapitation plus an application of lanolin.

LOT III.—Plants from lot II were used for further experimentation in wound response of control material. In each plant the second internode of the strongest axillary branch, which developed after the removal of the main stem, was decapitated. One half of these branches received no further treatment; the other half, an application of lanolin only.

LOT IV.—This lot of material was derived from an experiment in which bean plants were decapitated in the upper portion of the hypocotyl 7 days after planting. No further treatment was given, and collections were made at various intervals after decapitation. DOUTT (4), in her study of the anatomy of the bean plant, considered the upper portion of the hypocotyl similar in structure to the stem except for the tangential arrangement of the primary xylem (fig. 15*A*).

Observations

GROSS RESPONSES

LOT I.—The response of the two lots of plants treated with nicotine differed considerably in degree but not in kind. In lot I treatment with 2% nicotine induced a strong response, and a well-defined tumor was formed. The following description of gross response is based on observation of this first lot of material in which treatment was more effective.

The gross response of the decapitated stem to 2% nicotine indicates that only the uppermost and peripheral portions of the tip were obviously stimulated by treatment with nicotine. Visible response was initiated slowly. By the 3d day the apex showed a central depression in the pith and a slight flaring of the outer tissues. This outward flare extended down the stem less than 1 mm. By the 5th day the central cavity had deepened and was lined with injured or suberized pith cells. Occasionally, the xylem tissues at the margin of the cavity stood up as a slight ring or crown. Tissues outside this xylem ring were flared outward to a depth of 1.5 mm., and the stem tip had broadened

to twice its original diameter. The outermost tissues were then at right angles to the stem axis and gave the apex the appearance of a flat nailhead with a hollowed center. After the 7th day the stem below the cut surface changed little in appearance.

Proliferation of the flared stem tissues in the formation of the tumor was evident above the original level of the cut surface by the 5th day. The most rapid growth occurred between the 5th and 7th days and was followed by a period of gradual enlargement. In later stages the rate of growth appeared to diminish, but increase in size was noted until observations were discontinued at the end of 30 days. At this time a large tumor measured 13 mm. in transverse diameter and 8 mm. in length. In early stages of growth the proliferation was pale in color. Within a few days the tumor became light green, succulent in appearance, and finely tuberculate over the surface. Later growth was accomplished by maturation, and older tumors appeared firm and compact. Portions of the surface were brown where cells were suberized. In shape the tumor was flat across the top with the peripheral portions growing downward and closely encircling the stem (figs. 8*B*, 9*A*). These marginal areas showed the greatest growth and, in later stages, formed the larger part of the tumor. The central cavity was a characteristic feature and became partially filled or overgrown by proliferating tissues in older stages. The tumor was generally symmetrical as compared with the irregular tumors induced by such substances as indoleacetic acid. At no time was there evidence of the presence of root primordia.

Only brief observations were made of control plants grown in lot I. Decapitated stem tips, receiving no further treatment, occasionally developed small tumors. Those which received an application of lanolin showed consistently greater response, although no large tumors were formed. There was irregularity in the form of these tumors and in the degree of response. In one plant treated with lanolin a tumor which formed within 7 days after treatment was larger than some formed during 20 days' growth in other instances.

Lot II.—In this lot plants treated with 2% nicotine failed to show the vigor of response of the earlier experiment, and small irregular tumors were formed. Plants treated with 3% nicotine more nearly reached the degree of response evident in lot I.

Since particular emphasis was placed on control plants in lot II, observations of gross responses were more complete for this group. However, conditions for growth were apparently less favorable than with lot I. Simple decapitation of the second internode rarely induced tumor formation. The cut surface became brown and dry, and some shrinkage of the tissues occurred at the apex. Below the apex the tissues remained firm and green for a period of 3–4 weeks after decapitation. Following this period, yellowing became apparent in many stem tips, and they died back $\frac{1}{2}$ inch or more. In the lanolin-treated plants paling of the surface was marked, but the surface itself remained fresh in appearance. Generally a slight flaring of the tissues was detectable at the cut surface by the end of the 3d day. Further development was unpredictable. Often no more proliferation or callus formation was evident. In some plants tumors developed in 7–12 days (fig. 11*A*); in other plants a similar size was not attained until 27–31 days (fig. 11*B*). These tumors were irregular in form, and frequently only one sector of

the stem tip was involved (fig. 11*C*). No large tumors were observed.

Lot III.—In this lot the decapitated second internode of the axillary branch received treatment comparable with control material in the first two lots. However, the diameter of the axillary branch was greater and the tissues appeared to be more mature; the response was likewise greater in degree. Branches which were simply decapitated showed a darkening and drying of the surface; in those which received an application of lanolin, the cut surface appeared fresh and succulent under this covering. In both cases slight yellowing and flaring of the stem tip were detectable by the 2d day. Enlargement continued, and by the 5th–7th days it was clearly evident that the region of growth was a band of proliferating tissue lying between the outer tissues and the xylem. The xylem appeared as a distinctive ring or crown of darkened tissue, and the center of the stem was somewhat sunken and dried. Further growth changed the pattern but little. The band of proliferating tissue increased in width to form a broadly flared tumor which abruptly tapered downward to the diameter of the unaffected stem. Such tumors might reach a width of 6–7 mm. across and 4–5 mm. in length at the end of 2–3 weeks. There was no growth of peripheral portions of the tumor downward to encircle the stem, such as was noted for nicotine treatment.

Lot IV.—The response in this lot, composed of decapitated hypocotyls, was similar to that of the axillary branch of lot III. The broadly flared stem tip showed the band of proliferating tissues, the protruding crown of matured xylem elements, and a central cavity resulting from the disintegration of pith.

Histological responses

Nicotine.—The following detailed description of the histological responses of bean to nicotine is based upon a study of the first lot of plants. Nicotine, as applied in this experiment, induced responses only in the uppermost levels of the stem. The greatest proliferation occurred within 1 mm. of the surface of application; below this level the response diminished in degree and extended beyond 2–3 mm. only in the endodermis and in the cambium. Response varied from plant to plant, but these differences were more evident in the rate of reaction than in the pattern of response.

The epidermis and the cortical parenchyma were frequently injured at the cut surface and were limited in their response. Epidermal cells immediately below the injured ones might enlarge slightly and by the 3d day undergo one or two transverse divisions (fig. 1*A*). Further response consisted of the usual growth to allow for expansion of internal tissues. The downward extent of response in the cortical parenchyma was generally less than 0.5 mm. below the cut surface. Enlargement and proliferation of the cells were evident by the 3d day (fig. 1*A*) and continued through the 5th day. The degree of proliferation varied considerably from none at all to divisions in various planes in the upper cells (fig. 2). These divisions did not, however, contribute to the proliferation of a tumor above the cut surface.

The endodermis was more sensitive than the cortical parenchyma. By the 3d day (fig. 1*A*, *C*) endodermal cells down to about 1 mm. were enlarged, and proliferation was initiated in the uppermost cells. Maximum development was reached by the 5th day. Because of the outward flaring of tissues at the stem

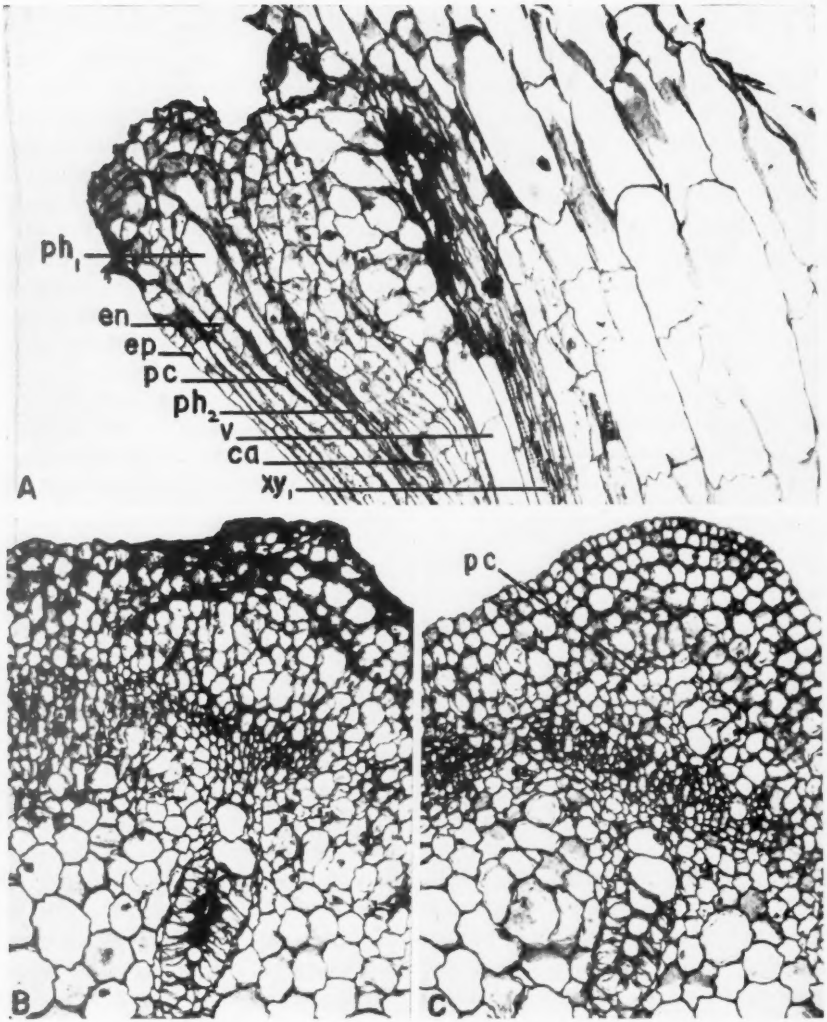

FIG. 1.—Decapitated second internodes, 3 days after treatment with 2% nicotine in lanolin. *A*, longisection: *ep*, epidermis; *en*, endodermis; *pc*, pericycle; ph_1, primary phloem; ph_2, secondary phloem; *ca*, cambium; *v*, vessel; xy_1, primary xylem. Initial proliferation of cells of endodermis, phloem parenchyma, and meristematic zone (cambium and its undifferentiated derivatives); a few divisions in epidermis; pericycle crushed; pith inactive. *B*, transection, 0.3 mm. below cut surface. Epidermis and cortex injured; pericycle over bundle crushed; parenchymatous cells of phloem and rays enlarged. Note characteristic elongation of xylem parenchyma encircling mature vessels. *C*, same stem as *B*, 0.5 mm. below cut surface. Endodermal cells enlarged radially. (Material for figs. 1–9 taken from lot I.)

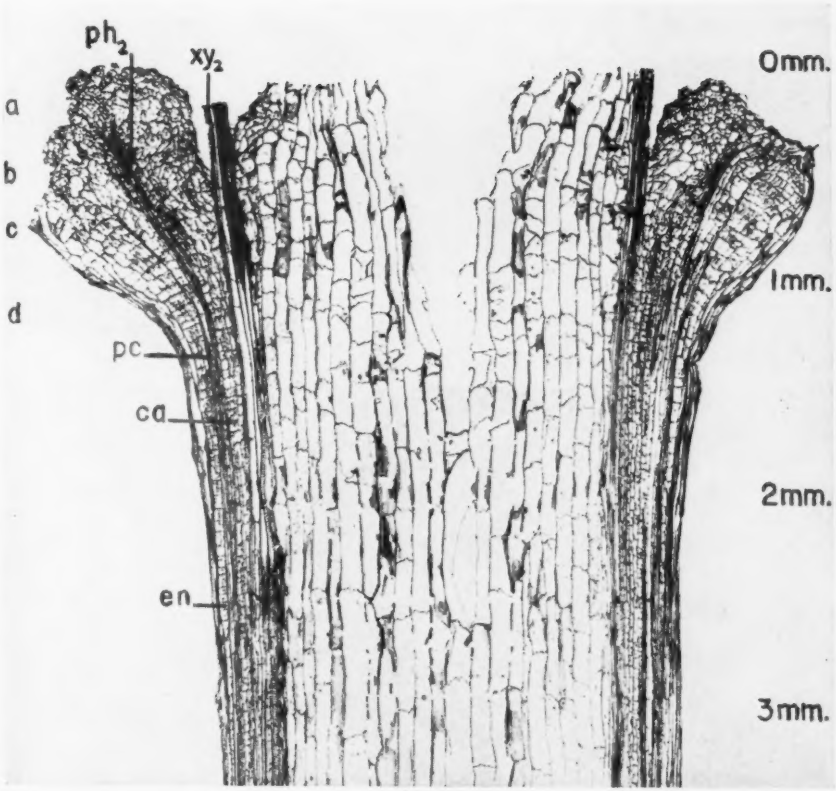

FIG. 2.—Longisection, 5 days after treatment with nicotine: xy_2, secondary xylem; *o mm.*, approximate level of cut surface as determined by severed vessels, outer tissues having been deflected downward. At apex proliferation of cortical parenchyma, endodermis, phloem parenchyma, and meristematic zone. Below cut surface enlargement and division of endodermal cells; pericycle crushed; secondary phloem sieve tubes and companion cells recognizable within short distance of cut surface. Meristematic zone very active and forming wedge-shaped area of proliferated tissue, which is torn from inactive secondary xylem at stem tip. Central pith disintegrating. Levels *a*, *b*, *c*, *d* similar to transections in figs. 3 and 4.

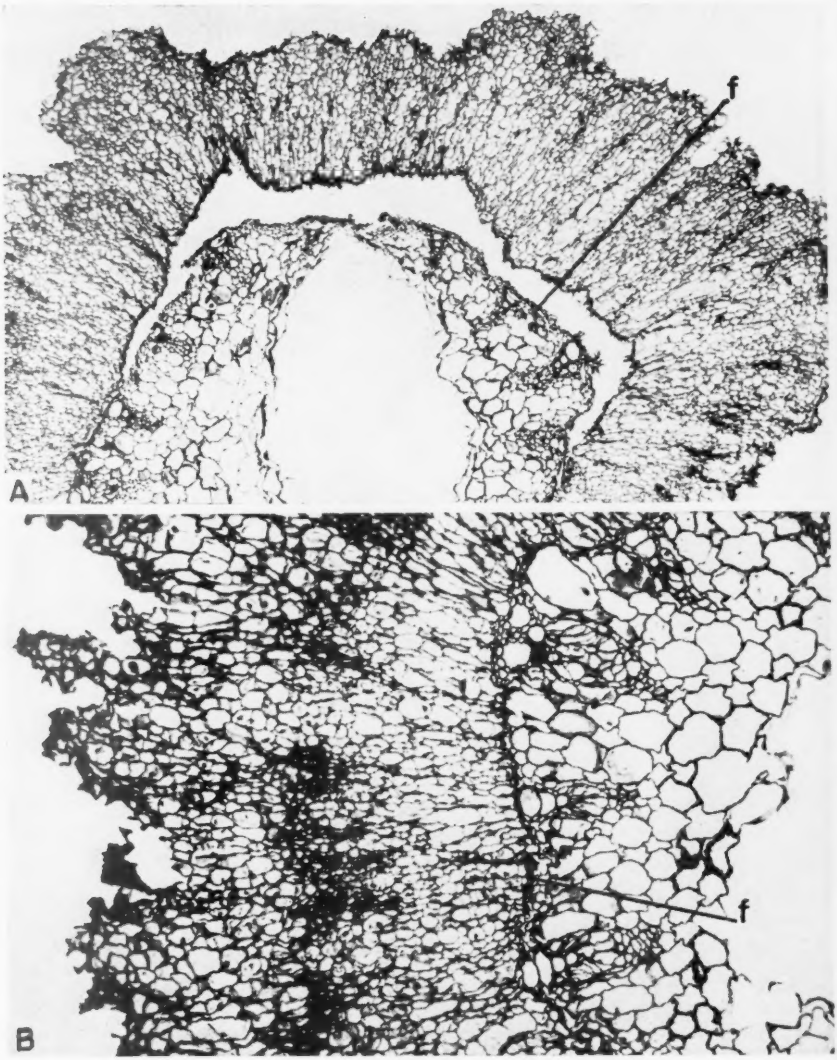

FIG. 3.—Transections, 5 days after treatment with nicotine. *A*, just below surface of application: *f*, secondary xylem layer of fibers. Outer segment proliferated mainly from meristematic zone; inner segment bordered on outer margin by fiber layer (*f*) and composed of inactive xylem, inner portion of ray, and pith tissues. Compare with level *a*, fig. 2. *B*, same stem, 0.5 mm. below cut surface. Marginal proliferated tissue derived from primary phloem parenchyma (tissues from pericycle outward deflected below this level); secondary phloem sieve tubes appearing as dark patches; wide band of proliferated tissue in meristematic zone; layer of secondary xylem fibers differentiated around stem; cells of inner portion of ray and xylem parenchyma adjacent to matured vessels much enlarged; central pith disintegrating. Compare with level *b*, fig. 2.

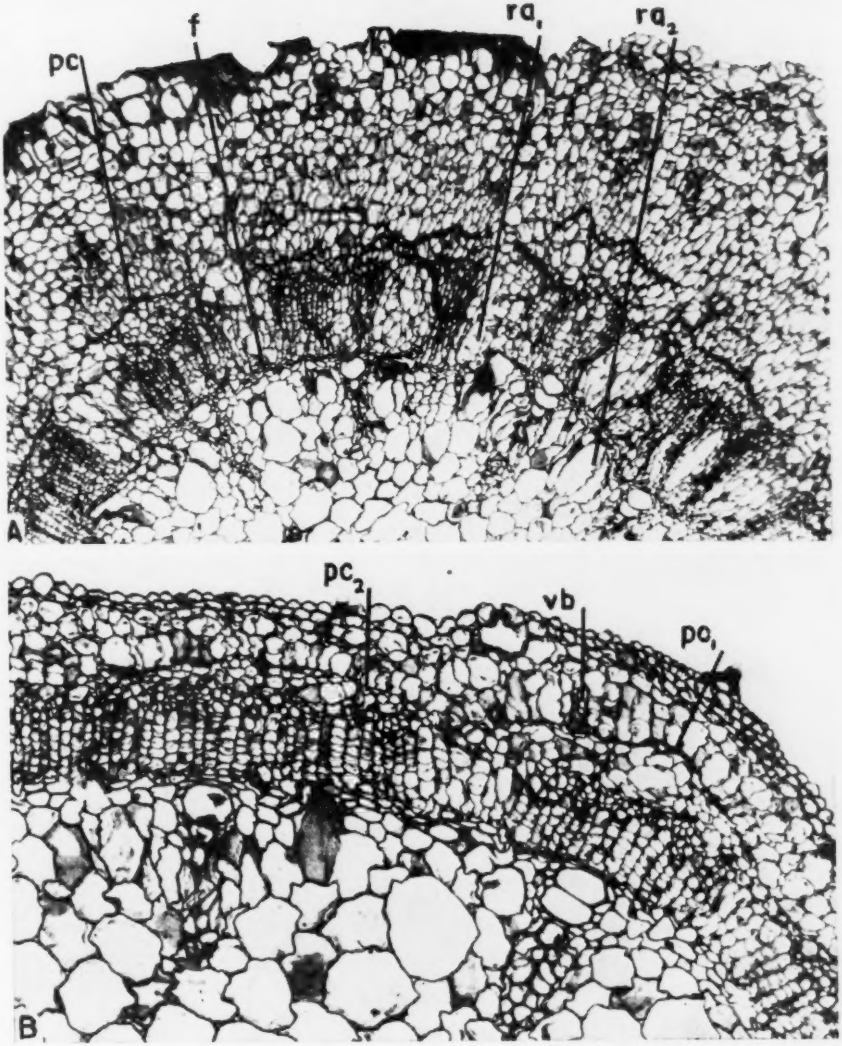

FIG. 4.—Transections, 5 days after treatment, same stem as fig. 3. *A*, 1 mm. below cut surface: *ra*, ray. Outermost cells enlarged cortical parenchyma; endodermal derivatives forming broad band because of outward deflection at stem tip; pericycle conspicuously crushed over bundles; wide band of proliferating cells in meristematic zone; outer portion of ray (ra_1) keeping pace with meristematic zone by means of cell enlargement and a few divisions; many cells of inner portion of ray (ra_2) greatly enlarged; layer of fibers interrupted across some rays. Compare with level *c*, fig. 2. *B*, same stem, 1.5 mm. below cut surface. Endodermal cells enlarged radially and a few divided tangentially; initiation of vascular bundle (*vb*) from derivatives of endodermis, occurrence infrequent. Pericyclic cells crushed (pc_1) or maturing as fibers (pc_2). Meristematic zone not so wide as at higher levels. Compare with level *d*, fig. 2.

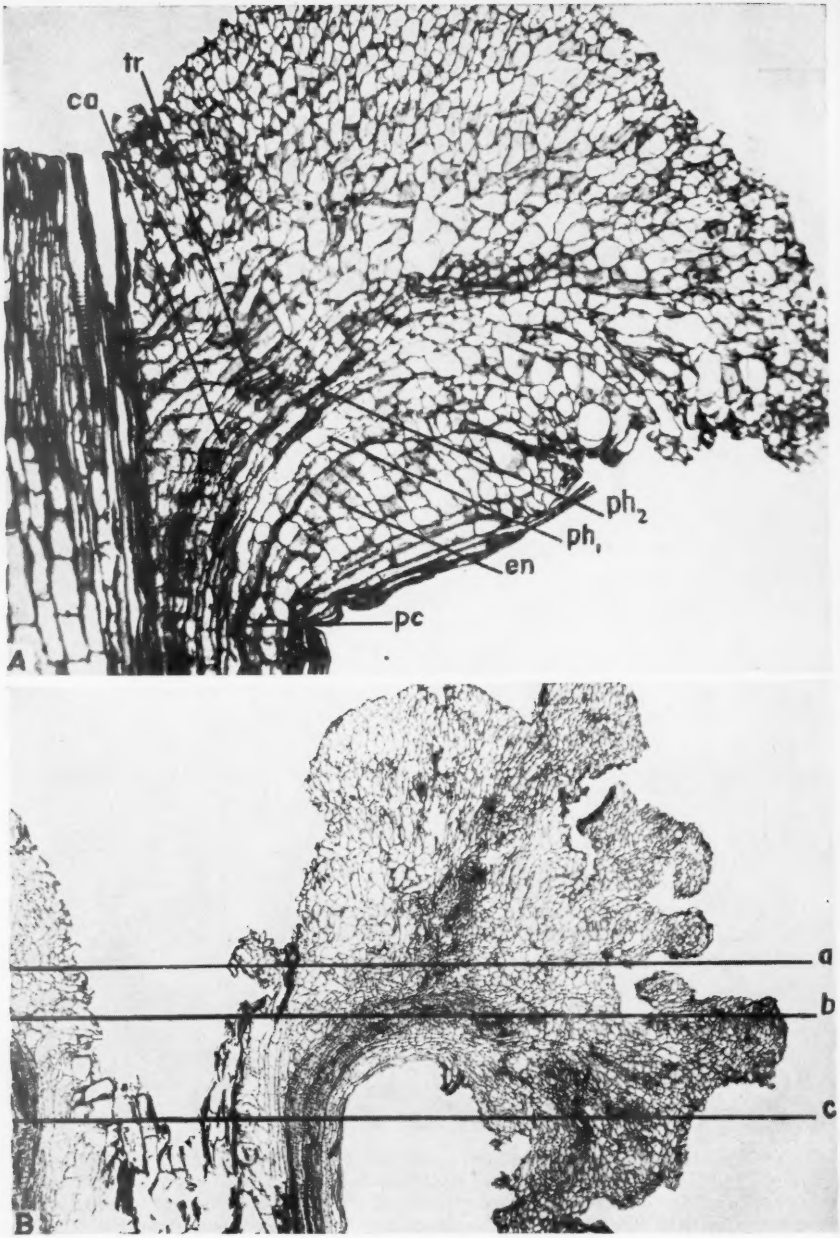

FIG. 5.—Longisections of decapitated stem treated with nicotine. *A*, 7 days: *tr*, tracheid. Early stage in development of tumor derived mainly from upward proliferation of meristematic zone; outermost tissues cut off by crushed pericycle. Beginning of maturation in derivatives of meristematic zone evident in differentiation of tracheids (*tr*) immediately centrad to cambium (*ca*). *B*, 16 days. More mature stage in development of tumor, showing layer of tracheids derived from cambium below original level of treated surface. Differentiation of vascular strands in tissue proliferated above treated surface. Levels *a*, *b*, *c* corresponding approximately to transections in figs. 6*A*, *B*, 8*B*, respectively.

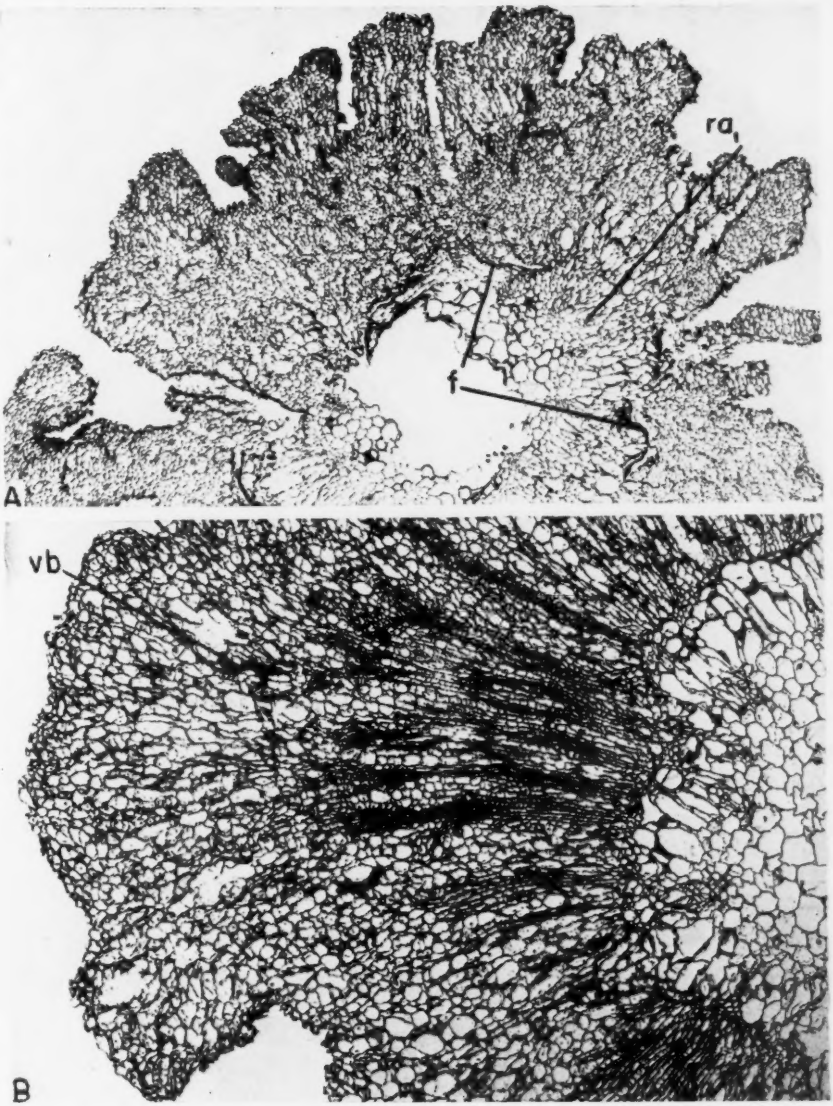

FIG. 6.—Transections of stem, 12 days after treatment with nicotine. *A*, at cut surface. Outer region highly meristematic periphery of tumor. Enlargement and slight proliferation of tissues centrad to secondary xylem layer of fibers (*f*); greater activity in rays lacking fiber layer (ra_1). Compare with level *a*, fig. 5*B*. *B*, same stem, 0.5 mm. below cut surface. *Pith inactive. Parenchyma cells of xylem and ray centrad to fiber* layer responding mainly with enlargement. Outside fiber layer radial rows of cells are vascular tissue differ*entiated from meristematic zone and deflected to horizontal position. Cells in remaining (outer) portion of* tumor maturing generally as parenchyma; some continuing activity to form vascular bundles (*vb*); others enlarged, divided, and collapsed; a few at periphery remaining meristematic. Compare with level *b*, fig. 5*B*.

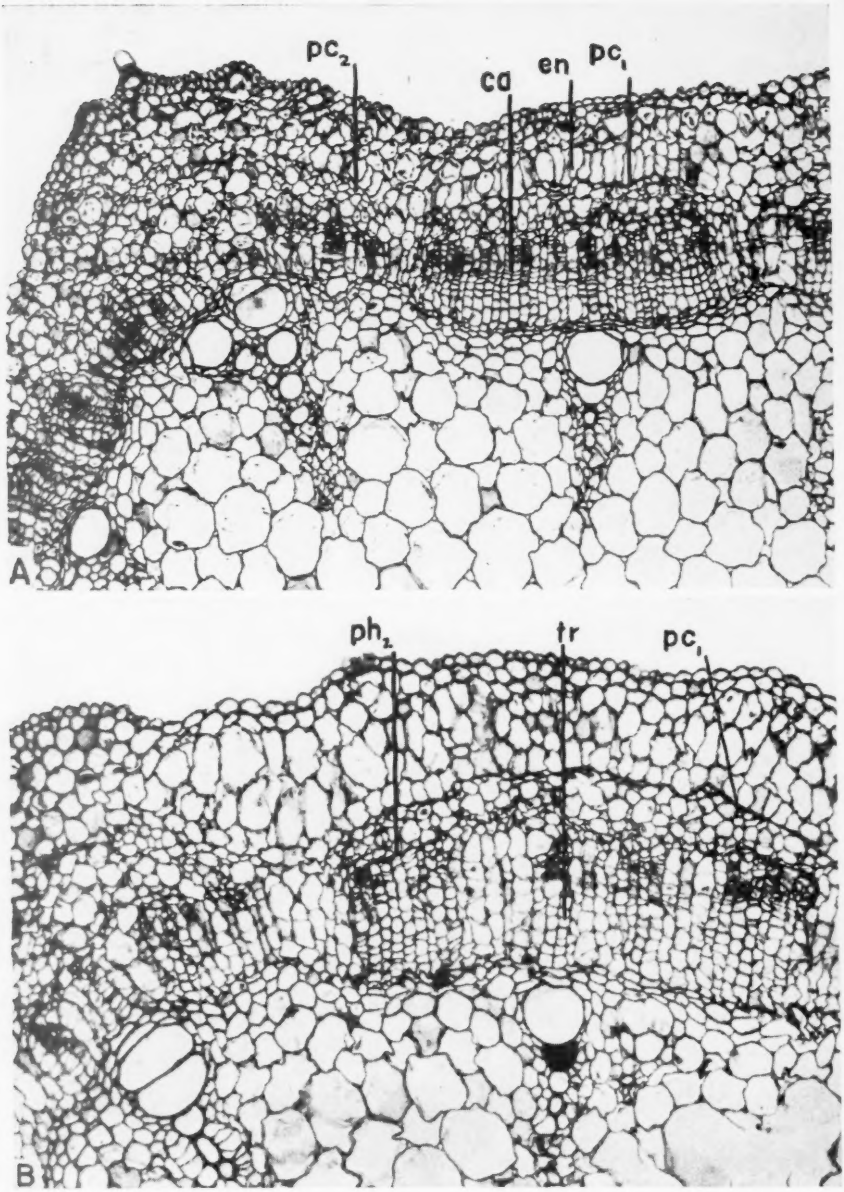

FIG. 7.—Transections of same stem as fig. 6, 12 days after treatment. *A*, 1 mm. below cut surface. Endodermal cells enlarged radially and a few dividing; many pericyclic cells crushed (pc_1) but some maturing into fibers (pc_2). Derivatives of meristematic zone differentiated as phloem and as xylem tracheids; narrow meristematic layer including cambium (*ca*); ray cells maturing as parenchyma. *B*, same stem, 1.5 mm. below cut surface. Response similar to that at 1 mm. Secondary phloem which matured before treatment is crushed (ph_2). Compare with transection of stem in fig. 8*B*.

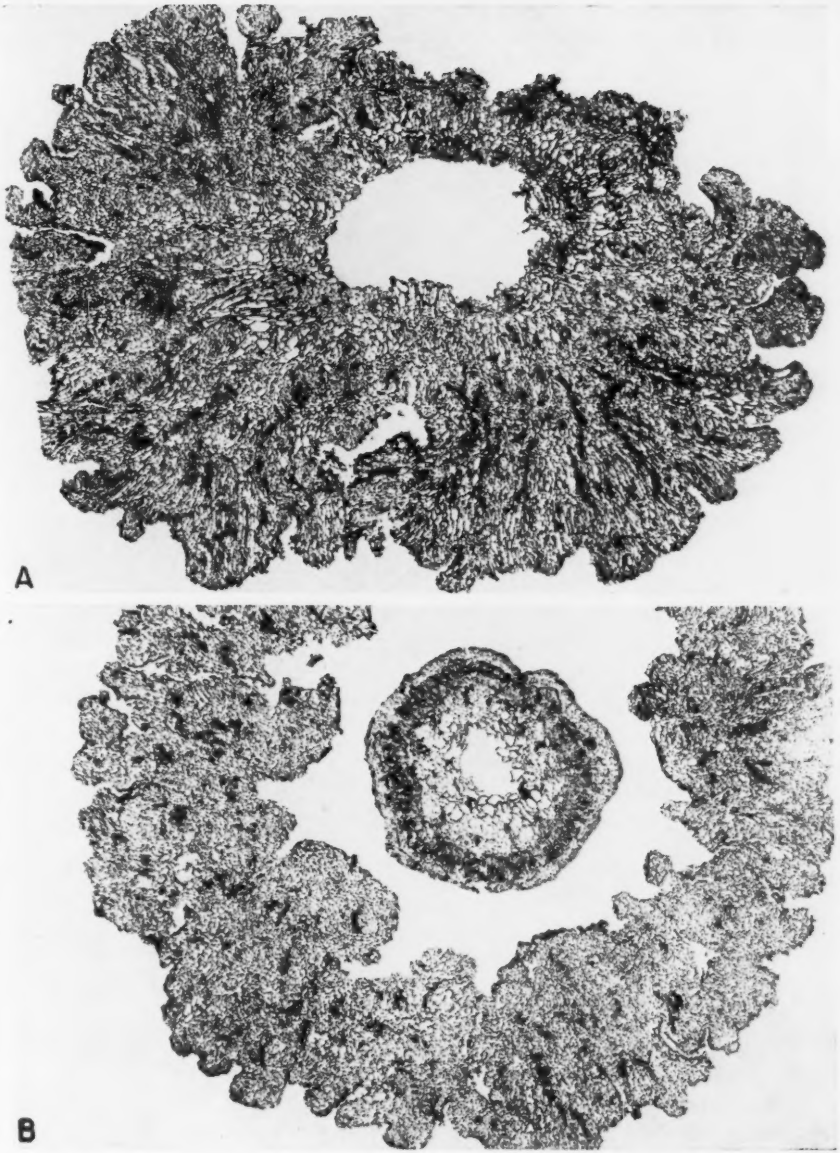

FIG. 8.—Transections of tumors, 19 days after treatment with nicotine. *A*, immediately below cut surface. Irregular proliferation at periphery results in tuberculate surface. Vascularization more advanced than in fig. 6*B*. *B*, 1.5 mm. below cut surface. Overhanging portions of tumor closely formed about stem axis in center. Compare with level *c*, fig. 5*B*. Tumor parenchymatous and highly vascularized (dark patches). Response in stem evident in wide band of tissues differentiated from meristematic zone.

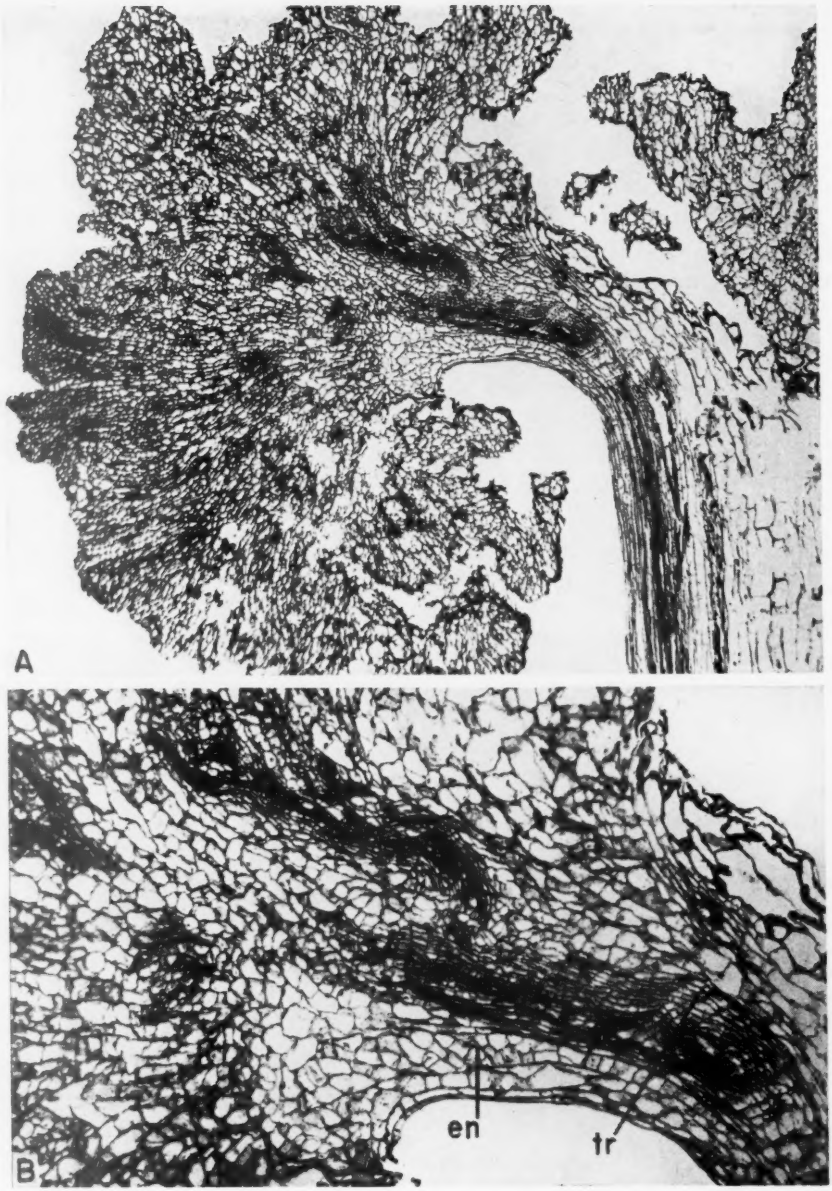

FIG. 9.—Longisection of apical tumor, 21 days after treatment with nicotine. *A*, growth of tumor in outward and downward directions; central cavity partially filled by overgrowth. *B*, detail of *A*. Vascular complex of tumor directly connected with vascular tissues in stem. Note general maturation of proliferated tissues as parenchyma, except for vascular bundle originally differentiated from meristematic zone (see fig. 5*A*) and now considerably enlarged through activity of cambium.

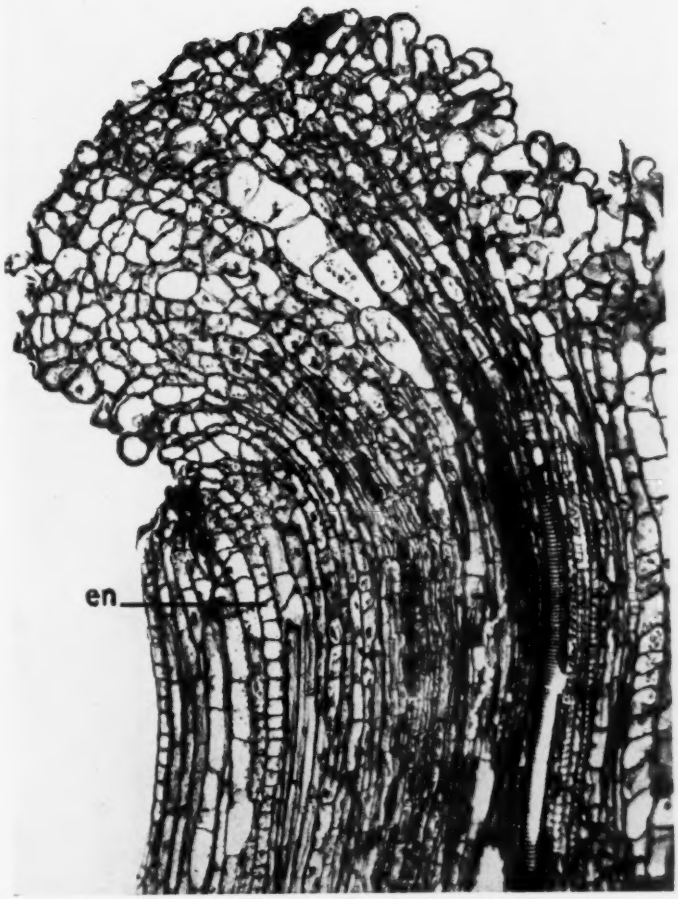

FIG. 10.—Decapitated stem from lot I, 20 days after treatment with lanolin only. At cut surface epidermis and cortical parenchyma injured; enlargement and slight proliferation of endodermal and pericyclic cells. Phloem parenchyma enlarged and proliferated; marked activity in cambium and adjacent undifferentiated cells (meristematic zone); segments of immature xylem vessel conspicuously enlarged. Superficial tumor formed mainly from surface proliferation of phloem, meristematic zone, and unlignified xylem tissues. Transverse phellogen forming in pith.

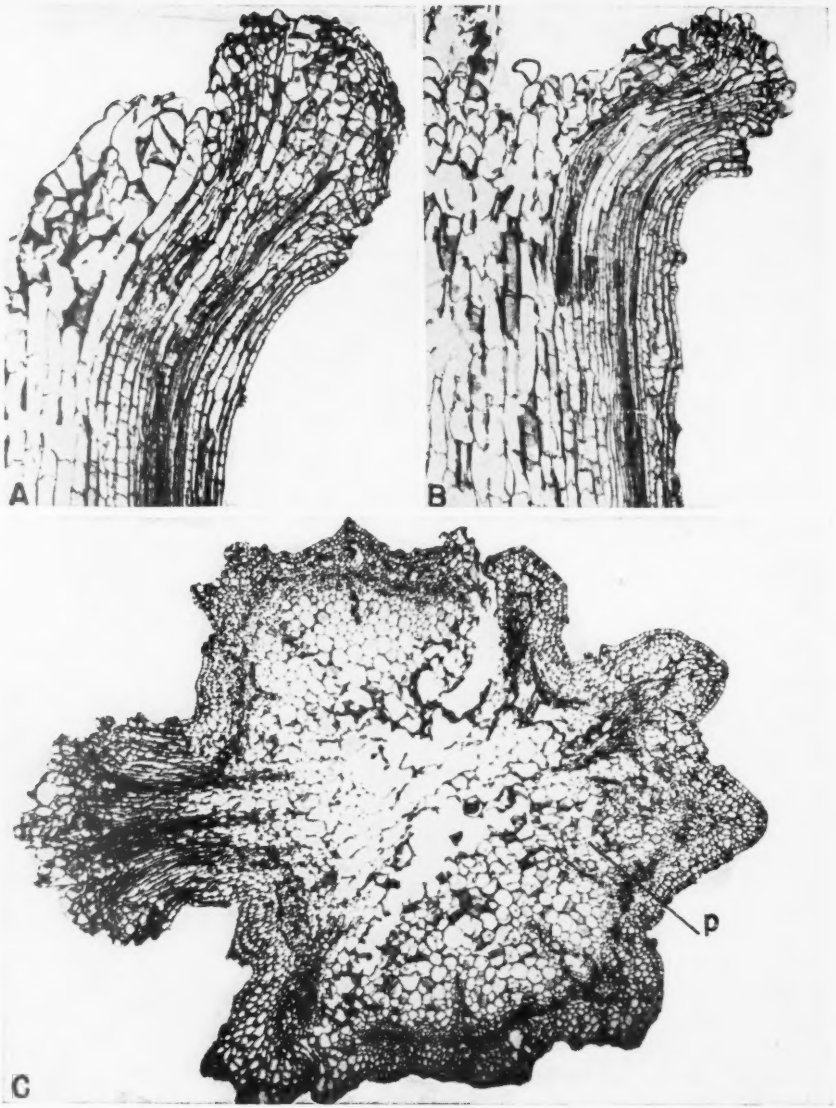

FIG. 11.—Decapitated stems from lot II, treated with lanolin only. *A*, longisection, 9 days. Development of superficial tumor similar to fig. 10. Transverse phellogen lacking. *B*, longisection, 27 days. Although considerably older, tumor similar to fig. 11*A* in extent of development. *C*, transection about 0.5 mm. below cut surface, 27 days: *p*, phellogen. Inequality of response over cut surface shown in proliferation of small callus at left, evidence of collapse of tissues at upper right, and formation of phellogen (*p*) in lower right sector. Note relatively immature stage in development of stem tissues and absence of secondary xylem fiber layer, characteristic of material in lot II.

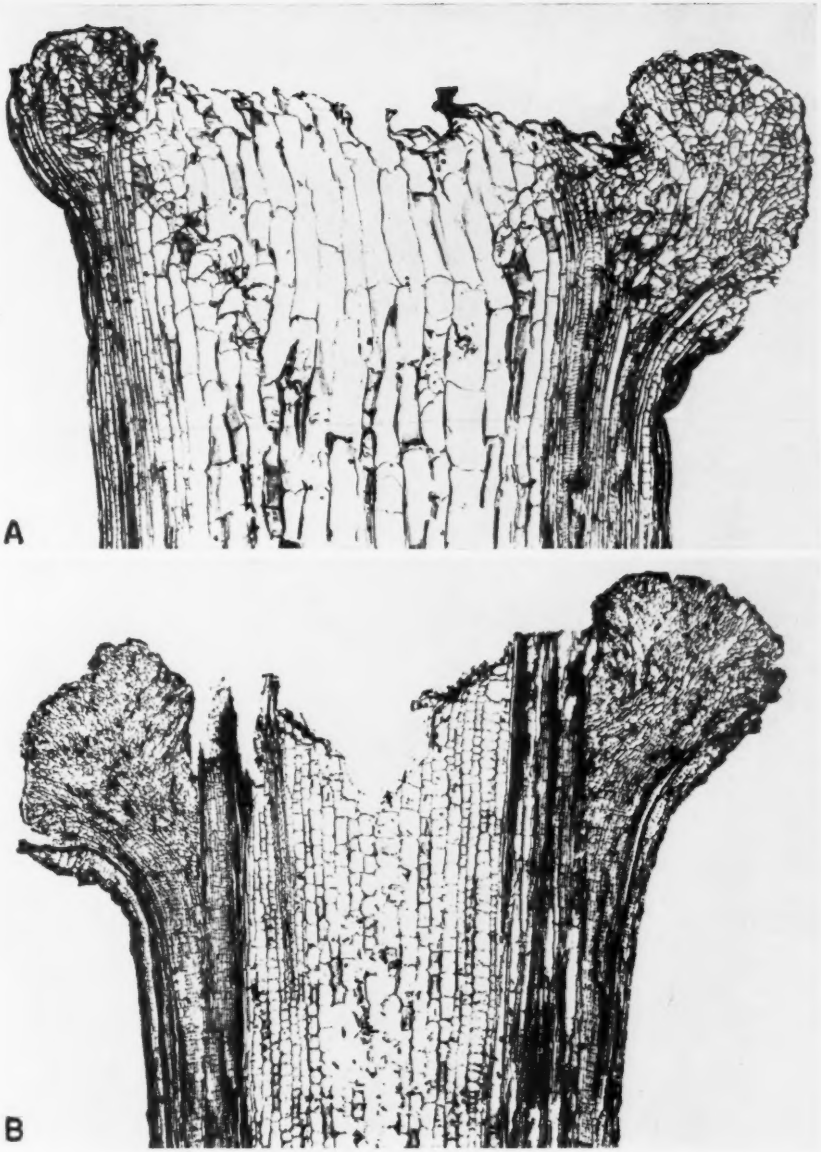

FIG. 12.—*A*, longisection of second internode from lot I, 7 days after decapitation, cut surface untreated. Superficial tumor derived mainly from activity in meristematic zone. Comparable in development to fig. 10. Traces of transverse phellogen. *B*, longisection of axillary branch from lot III, 9 days after decapitation, cut surface untreated. Proliferation of callus mainly in meristematic zone lying between well-developed secondary xylem and secondary phloem. Some proliferation in endodermis. As compared with *A*, greater proliferation of callus associated with greater activity of stem.

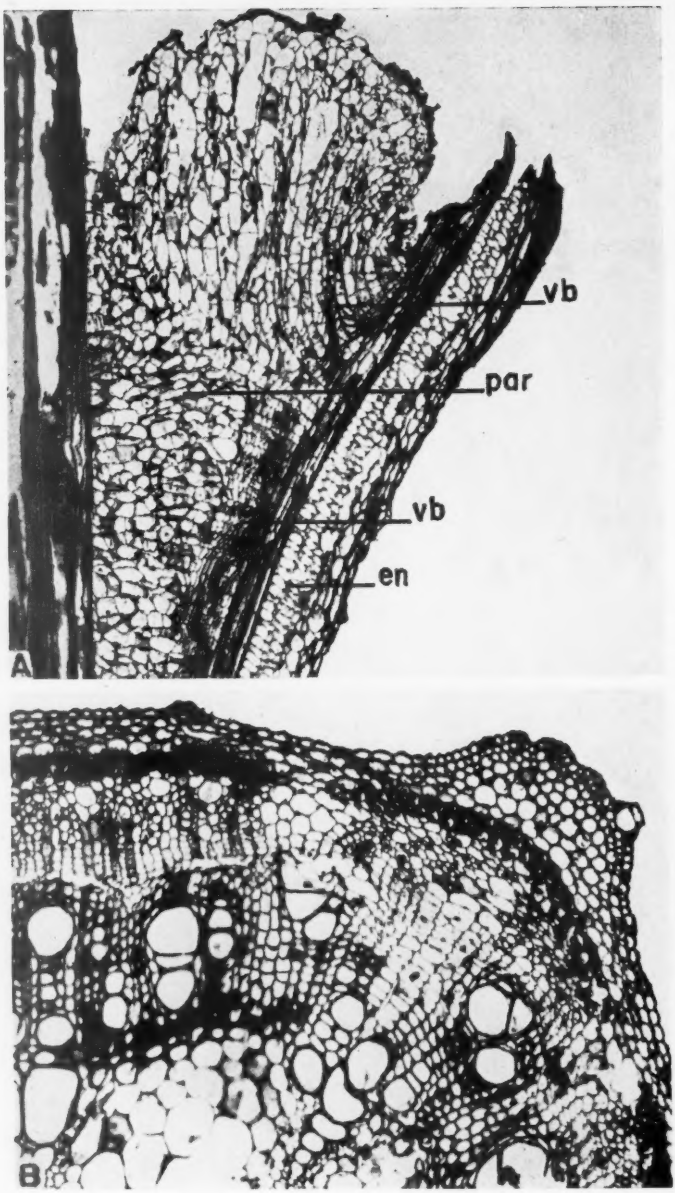

FIG. 13.—Axillary branches from lot III. *A*, longisection, 12 days after decapitation and treatment with lanolin only: *par*, parenchyma. Outer tissues inactive, except for slight proliferation in endodermis. Lignified pericyclic fibers showing no response. Proliferated, wedge-shaped meristematic zone lying between mature secondary phloem and secondary xylem and composed of vascular strands (*vb*) and parenchymatous tissue (*par*). *B*, detail of transverse section of axillary branch at time of decapitation. Mature stage of development shown in secondary tissues and heavily lignified pericyclic fibers. Compare with fig. 11*C*.

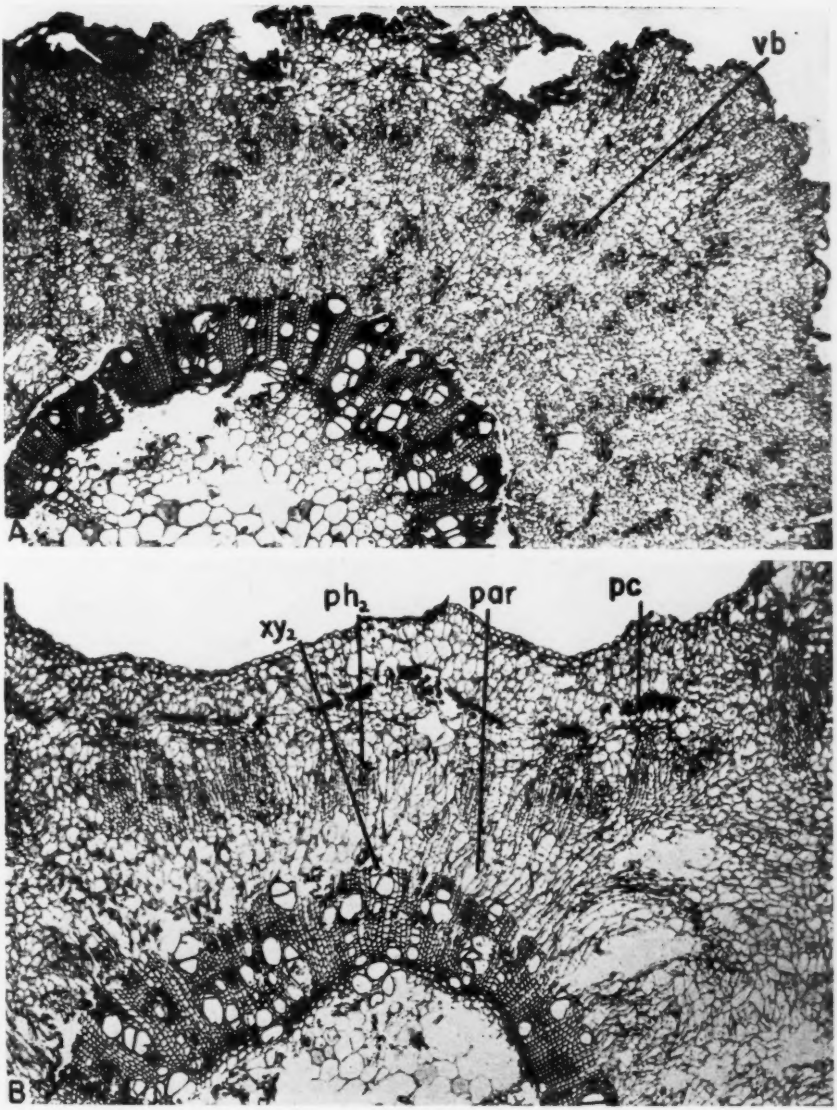

FIG. 14.—Transections of decapitated axillary branch from lot III, 9 days after treatment with lanolin. *A*, near cut surface through apical portion of callus. Outer tissues proliferated from phloem parenchyma and meristematic zone. Vascularization resulting in complex of bundles (*vb*). Inner cylinder consisting of secondary xylem formed before treatment and disintegrating pith. *B*, same stem, at level comparable to lowest level of fig. 13*A*. Little response in outer tissues; derivatives of meristematic zone (between ph_2 and xy_2) matured as vascular tissue or as parenchyma (*par*).

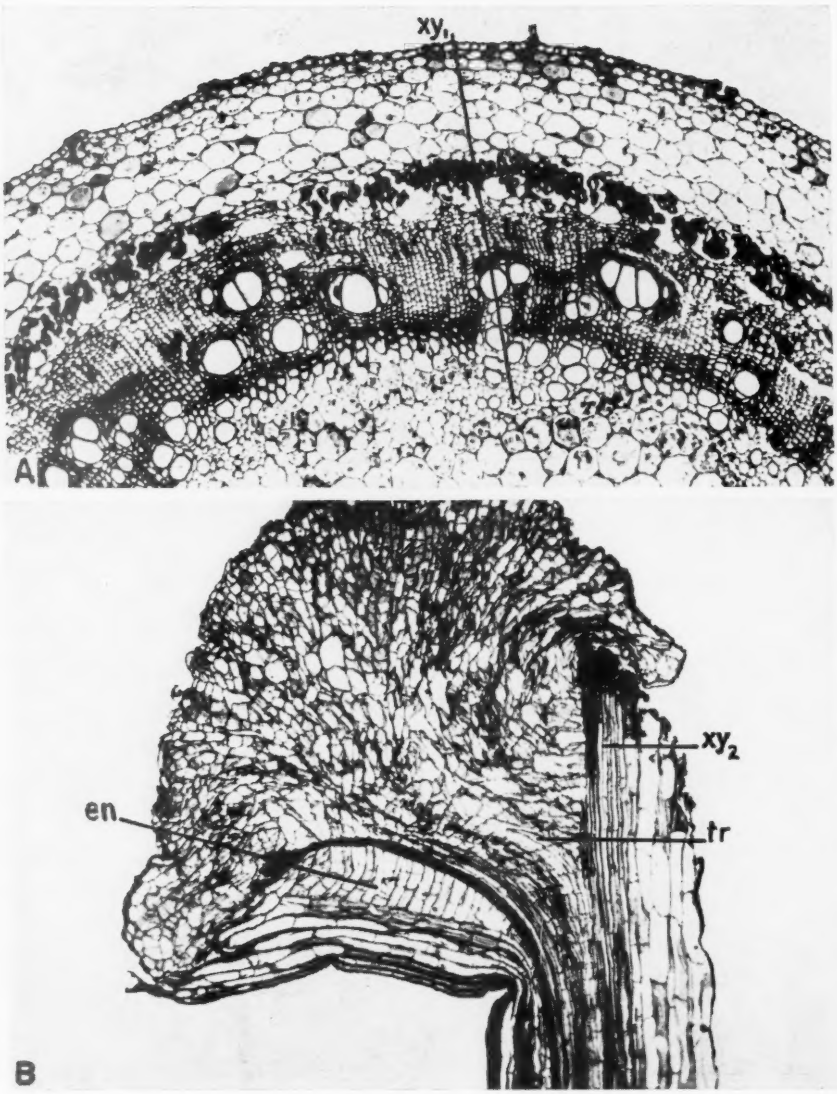

FIG. 15.—*A*, transection of hypocotyl below cotyledonary node. Mature stage of development, showing stemlike arrangement of tissues except for tangential arrangement of primary xylem (xy_1). *B*, longisection of hypocotyl from lot IV, 25 days after decapitation, cut surface untreated. Marked proliferation in endodermis; otherwise, development of callus similar to that in other control material. Note similarity to fig. 5*A* of material treated with nicotine.

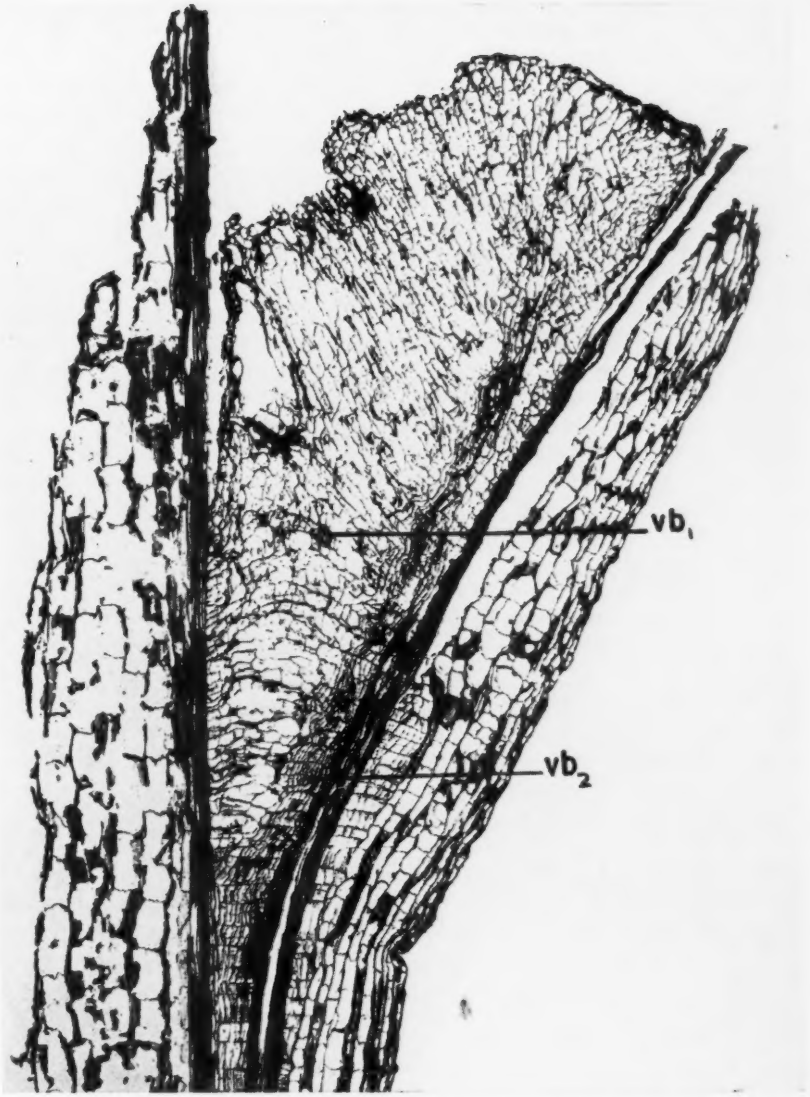

FIG. 16.—Longisection of hypocotyl, 15 days after decapitation, cut surface untreated. Wedge of proliferated tissue derived from meristematic zone lying between phloem and xylem formed in axis before treatment. Broad, upper portion proliferating to form outward flaring callus. Derivatives in proliferated area matured as: parenchyma; small vascular bundles (vb_1) scattered among parenchymatous cells; a well-defined vascular bundle (vb_2) formed along outer margin of callus and continuous with vascular tissues of axis.

tip, the orderly pattern of response is most clearly seen in longitudinal sections (figs. 2, 5*A*). Just below the cut surface was a zone of cells dividing in irregular planes. At lower levels divisions were limited to the tangential plane, and the derivatives appeared in successive rows, two to six cells deep. At still lower levels of response (between 2–3 mm.) was a region of simple radial enlargement. The endodermis did not form a highly meristematic layer and only infrequently initiated vascular tissue (fig. 4*B*). Generally, the derivatives matured as parenchyma. Little or none of the tumor was derived from the endodermis, although in some plants a few dividing cells at the cut surface might be associated with the proliferation of the much more active inner tissues (fig. 9*B*).

The response of pericycle to treatment with nicotine was influenced by the thickness of the cell walls at the time of decapitation. If the uppermost pericyclic cells were immature at this time, they might enlarge and become indistinguishable from adjoining tissues or they might be crushed beyond recognition (fig. 2). If these cells were partially matured, they were frequently crushed and by the 5th day appeared as a conspicuous dark line in both longitudinal (fig. 2) and cross sections (figs. 4*A*, *B*, 7*A*, *B*). In the latter, this crushed pericycle was clearly evident between 0.5 and 1.5 mm., especially over the bundles. At lower levels some of the pericyclic cells persisted uncrushed, and their walls thickened slightly. At increasing distance from the cut surface there was the usual maturation of fibers. The pericycle appeared to take no part in the formation of the tumor. Moreover, when the pericycle was crushed in the upper levels, outer tissues might be cut off from participation in tumor formation (fig. 5*A*).

Response in the upper levels of primary phloem parenchyma consisted of cell enlargement and divisions in various planes. This proliferation was like that of the cortical parenchyma, but it was greater in amount and contributed to the outward flaring of the stem tip. Enlargement and proliferation continued through the 5th day, the response finally extending to about 1.5 mm. below the cut surface (fig. 2). The derivatives matured as parenchyma. At the cut surface continued proliferation produced a small part of the peripheral portion of the tumor (fig. 9*A*, *B*).

Cells of the secondary phloem parenchyma, especially those in the phloem rays, responded in a manner similar to the primary phloem parenchyma. Enlargement and proliferation of the cells occurred in the upper levels and were completed by the 5th day. Sieve tubes and companion cells showed little activity and could be readily identified to within a few cells of the cut surface (fig. 2), although these sieve-tube groups might be disarranged by the activity of intervening phloem rays. As a secondary consequence of the proliferation of tissues centrad to the secondary phloem, sieve tubes and companion cells matured previous to decapitation might be crushed. This crushing became noticeable about the 9th day and, by the 12th day, resulted in patches of collapsed cells (fig. 7*B*). These were distinct at 1 mm. and extended to 2.5 mm. below the cut surface. Derivatives of secondary phloem became indistinguishable from those of the primary phloem in forming peripheral portions of the tumor above the cut surface.

The greatest degree of proliferation in response to treatment with nicotine was in the zone between mature secondary phloem and mature secondary xylem,

a region consisting of the cambium and the undifferentiated cells lying on either side of the cambium. Proliferation of these tissues resulted in an area of active tissue which is designated the meristematic zone. Cells of those portions of the interfascicular rays abutting the cambium and its derivatives likewise proliferated so that this zone was continuous about the stem (figs. 3*A*, 4*A*).

Proliferation in the meristematic zone was quickly initiated after treatment. By the 3d day cells in the uppermost levels had undergone divisions in various planes and had formed an irregular mass of tissue (fig. 1*A*). Between 0.5 to 1.5 mm. below the original level of the cut surface the undifferentiated derivatives were enlarged, often in a radial direction, and there was an increased number of cambial derivatives. By the 5th day a broad region of highly meristematic, generally proliferating, tissues had been formed at the stem tip (fig. 2). At lower levels this became a wide zone of radially arranged, apparently multinucleate cells. During the period of greatest proliferation successive divisions appeared to be so rapid that cell wall formation did not keep pace with nuclear division. When wall formation was completed later, the multicellular nature of the zone was clearly evident. With increasing distance from the cut surface the number of cell divisions and the width of the meristematic zone diminished. This zone was conspicuously wide at 1 mm. (fig. 4*A*) and much narrower (fig. 4*B*) but still evident at 2 mm. Activity in the meristematic zone resulted in the formation of a wedge-shaped ring of tissue (fig. 5*A*, *B*) which contributed largely to the outward flaring of the stem tip.

Below the original level of the cut surface maturation of cells in the meristematic zone began in 6 or 7 days and was first evident in the formation of relatively small, narrow, slightly elongated, reticulately thickened tracheids. Differentiation, as observed in longisections, was initiated along the curve of the outward flaring of the stem tip (fig. 5*A*). Outside this initial layer of tracheids the cells remained meristematic and formed an active and readily identified cambium. Cambial divisions resulted in additional tracheids so that by the 16th day a well-defined layer of tracheids was formed, often ten cells deep (figs. 5*B*, 9*A*, *B*). Outside the cambium a narrow layer of new phloem was differentiated (fig. 7*A*, *B*). At the lowest levels of response this layer of tracheids was adjacent to the xylem formed before treatment, and the newly formed xylem and phloem tissues merged with the new secondary tissues of unaffected portions of the stem. At the upper levels the proliferated tissues centrad to the tracheid layer generally matured as parenchyma. In the apical portions of the meristematic zone which proliferated above the original level of the cut surface, those cells near the periphery remained meristematic and proliferated actively to form the major part of the tumor.

Response of the xylem was dependent upon the formation and maturity of the cells at the time of decapitation. The degree of maturity varied from stem to stem and within a single stem. The first layer of secondary xylem differentiated in the bean stem consisted of fibers (4). In some of the treated stems this layer of fibers was matured almost continuously around the stem; in others it was interrupted across the rays or lacking over wide sectors (fig. 6*A*, *B*). Mature fibers and lignified vessels formed the ring or crown of xylem at the cut surface (fig. 2). These cells and elements showed little change following decapitation, except

that the very dark color resulting from the staining process suggested some change in chemical constitution. Because of differential activity, the tissues of the meristematic zone were frequently torn from this xylem layer. In the early stages of response this split might extend as far as 0.5 mm. below the cut surface (figs. 2, 3*A*). If the vessel segments, tracheids, and fibers were immature at the time of treatment, they might participate in the general apical proliferation. Somewhat below the cut surface (about 0.5 mm.) these immature elements often became enlarged; their cell walls were greatly stretched and in places might even be ruptured (figs. 3*B*, 4*A*). Cells of the xylem parenchyma adjacent to the vessels often enlarged, and in transection they formed a striking pattern encircling the vessels (fig. 1*B*, *C*). Frequently, the nucleus lay at the end of the parenchyma cell nearest the vessel. Response in the xylem parenchyma was evident by the 3d day. It might extend downward to 2 mm.

The interfascicular ray may be divided at the layer of fibers into an outer and an inner portion, the outer portion responding as part of the meristematic zone. At the cut surface the cells proliferated, and their derivatives became indistinguishable from those of the adjoining tissues (fig. 3*A*). At slightly lower levels, however, cells in this outer portion of the ray enlarged, especially in a radial direction. They might undergo a few tangential divisions, but no interfascicular cambium was formed. The derivatives matured as parenchyma. The large size of these cells distinguished the rays from other tissues proliferated in the meristematic zone (fig. 4*A*).

Response in the inner portion of the ray varied with the presence or absence of a layer of fiber. In general, when the fiber layer was present, only a few ray cells enlarged and underwent occasional divisions (figs. 3*B*, 4*A*). If the fiber layer was absent, the ray cells responded more generally with enlargement and division, especially those cells in close proximity to the meristematic zone (fig. 6*A*). The response in the inner portion of the ray appeared by the 3d day, and little change took place after the 5th day. The downward extent of the response varied; generally it was about 1 mm. At the cut surface the cells were frequently injured in treatment and did not participate in the formation of the tumor.

The cells of the pith showed no tendency to proliferate but began to collapse near the cut surface by the 3d day. This effect extended downward, and by the 5th day disintegration commenced in the center of the stem (fig. 2). Collapse of the pith resulted in a central depression characteristic of the gross aspects of the tumor. In older stages collapse of the pith might reach a depth of 1.5 mm. The central depression frequently became partially filled with tissue proliferated from the meristematic zone (fig. 9*A*).

The tumor was derived from the following tissues: a slight amount from the endodermis; a small part, mainly in the lateral portion, from primary and secondary phloem parenchyma; and a major portion from the highly responsive meristematic zone. That portion of the tumor which was above the cut surface resulted from the proliferation of these tissues. Upward growth was not distinct until the 5th day (fig. 2) and was pronounced by the 7th day (fig. 5*A*). The tumor increased in size for several weeks. The peripheral portions were small-celled and meristematic. In inner portions the tissue was parenchymatous (fig. 9*A*). Some cells might become very large and crush adjacent cells. The large cells might un-

dergo division, but often the walls were fragile and collapsed (fig. 6*B*). Vascularization was evident by the 12th day and was mainly a differentiation in apposition to the numerous vascular strands from the stem below. A complex system of branching vascular strands resulted. These were composed of short reticulate tracheids, narrow cambial-like cells, and small phloem-like elements. As maturation of the tumor proceeded, vascularization occurred in the peripheral portion, marginal cells lost their meristematic character, and exterior cells became suberized (figs. 5*B*, 8*A*, 9*A*, *B*).

No root primordia were observed in the material studied. The endodermis, the rays, and adjacent tissues did not reach a degree of proliferation and organization suggestive of the development of root histogens.

Histological examination of the stem tips treated in lot II confirmed early observation of gross material. The tumors formed as a result of treatment with 2% nicotine were small, while those formed after treatment with 3% nicotine were larger but not so vigorous as tumors in lot I. Both concentrations of nicotine produced tumors histologically similar to those already described in detail for lot I. However, associated with the weaker response of lot II was a lesser maturity of the stem tissues. This difference somewhat modified the appearance of proliferation at the stem tip. Especially significant was the absence of strongly developed xylem. The meristematic zone was not so clearly set off, and the ring or crown of protruding xylem elements was lacking at the cut surface.

CONTROL.—In the plants considered as controls the character of the response was consistent, despite differences in age and portion of plant involved (figs. 11*C*, 13*B*, 15*A*) and variations in time and extent of response. The limited proliferation observed in the stem tips of lots I and II (figs. 10, 11*A*, *B*, 12*A*) represented the initial stages of callus formation. In lots III and IV (figs. 12*B*, 13*A*, 15*B*, 16) proliferation advanced beyond these initial stages to the formation of a well-developed callus or tumor. The presence of lanolin on the cut surface tended to induce a greater degree of response than that which occurred in simply decapitated stem tips of comparable material. Because wound response in the control material was markedly similar to response following treatment with nicotine, a brief description of the former will suffice.

In the outer tissues of the stem the response was slight. The epidermis was injured, and the uppermost cells might undergo transverse divisions or otherwise show adjustment to internal expansion. The uppermost cells of the cortical parenchyma might be injured or might proliferate slightly (fig. 16). The endodermis varied considerably in the degree of response. It might show almost no change, particularly in cases of weak response of the stem tip (figs. 10, 11*A*, *B*, 12*A*). In other plants there might be characteristic radial elongation and tangential divisions of the endodermal cells. A layer several cells deep might result from these divisions (figs. 13*A*, 15*B*, 16). With very vigorous response a highly meristematic tissue was formed. The pericycle followed the pattern described for the nicotine treatment: at upper levels it might proliferate slightly (fig. 10) or be crushed (fig. 12*B*); at lower levels it matured as the usual fibers (fig. 14*B*).

The vascular and ray tissues responded in much the same manner as in plants treated with nicotine. At upper levels the parenchymatous cells of the primary and secondary phloem enlarged and prolifer-

ated to form the outer portions of the tumor or callus (fig. 14*A*). Matured sieve tubes and companion cells changed little and were readily identified in the enlarging stem tip. Again the activity of greatest significance in the formation of the wound callus or tumor was that of the cambium and adjacent undifferentiated tissues. The resulting meristematic zone was most active at the upper levels of the stem tip and gradually narrowed at lower levels to blend indistinguishably with normal secondary growth (fig. 16). As seen in longisection, this meristematic zone appeared as a wedge of proliferated tissue, with its narrow point lying between mature secondary phloem and secondary xylem and its broad portion forming the outward flaring callus or tumor (fig. 16). Maturation of this proliferated tissue occurred as in nicotine treatment. At levels below the original cut surface a cambium could be identified toward the outer or centrifugal side of the wedge. Activity of this cambium resulted in the formation of new phloem on the outside and new xylem tracheids on the inside. These tracheids were characteristically short, reticulately lignified, and often radially arranged. The remaining cells of the meristematic zone matured as parenchyma or differentiated scattered vascular bundles. The periphery of the tumor above the original level of the cut surface (fig. 15*B*), however, tended to remain meristematic and to continue proliferation with proportionate increase in the size of the tumor.

The pith and the xylem formed previous to decapitation showed little activity. Lignified xylem elements at the stem tip protruded as the ring or crown visible in gross observations (figs. 13*A*, 16). Immature or parenchymatous cells of the xylem, especially in younger stems, might enlarge and proliferate slightly near the cut surface (fig. 10). The cells of the pith generally collapsed following decapitation, although in younger stems a transverse phellogen might form (figs. 10, 11*C*, 12*A*).

Discussion

In the present experiment the response of control bean plants to decapitation conformed to the description of wound response and callus formation summarized by KÜSTER (11) for plants in general. A dominant role was assigned to the cambium in the formation of a wedge-like ring of callus tissue for which KÜSTER used the term "Lohden-wedge" as originally given by HARTIG. KÜSTER stated that the activity of the pith and phloem was variable, although in some plants the latter was scarcely less active than the cambium. He likewise noted the inequality of response over different parts of the cut surface. SCHILBERSZKY (19), in more detailed studies of wound response in species of *Phaseolus*, also described the formation of a strong callus from the region of the cambium. Under special conditions of wounding, he recorded the formation of a meristematic layer derived from the endodermis and producing well-developed extrafascicular bundles. This degree of development was not attained in the specimens of the present study.

In both the plants treated with nicotine and those considered as controls, the degree of response differed considerably from lot to lot. These differences are ascribed to factors in the environment or to the nutritive status of the plant. Both SCHILBERSZKY and KÜSTER have noted the significance of such factors as moisture and food supply in causing variation in the degree of natural wound response. FLINT and MORELAND (6) reported striking differences in natural wound tumors

under varying conditions of humidity and light. The experiments of BAUSOR (1), BEAL (2), and MITCHELL and BROWN (14) have shown the interrelationship between the reaction to growth-regulating substances and the nutrition of the plant. In the present experiment environmental factors, such as light, were more favorable for the nutrition and growth of plants in lot I than for those in lot II. The significance of age was apparent among control plants, in which response was greater in the axillary branch and hypocotyl than in the young stem tip. On the basis of histological observations of all the lots, greater response was associated with vigorous growth and a greater maturity of stems.

It is difficult to evaluate the position of nicotine as a growth-regulating substance. Nicotine in the form of nicotine sulphate has been repeatedly applied in great dilution as an insecticide with no apparent effect on the plant. Experiments in which nicotine has been supplied to the plant in nutrient solution have produced results which have ranged from toxicity to stimulation. As a result of experiments with rooting, TRAUB (21) placed nicotine in the same category as the highly stimulative indolebutyric acid. Yet in the series of histological studies on bean, nicotine differs markedly from the response described by HAMNER and KRAUS (7) for indolebutyric acid, both in the character of the tumor and in the absence of root primordia. Root formation also separates response with nicotine from response with indoleacetic acid, naphthaleneacetic acid, α-naphthalene acetamide, and some substituted phenoxy compounds. Response with nicotine was more like that with tryptophane and tetrahydrofurfuryl butyrate, in which root primordia were absent, and with phenylacetic acid, in which root formation was infrequent. However, root formation cannot be considered an absolute criterion in evaluating responses. Not only has TRAUB recorded root formation with nicotine—under different circumstances and with different plant material—but also recent observations with various concentrations of indoleacetic acid (16) indicate that the presence or absence of root primordia may be dependent upon the concentration of growth-regulating substance used.

The pattern of response induced by various growth-regulating substances, the degree of proliferation, and the type of maturation in the tissues separate response to one growth-regulating substance from response to another substance. Particularly significant are the tissues showing the greatest degree of activity. With nicotine this was the area of the cambium, designated the meristematic zone. The pith was highly responsive to indoleacetic and indolebutyric acids and less so to naphthaleneacetic acid. All three resulted in conspicuous activity in the endodermis, the phloem, and the rays. Application of α-naphthalene acetamide caused a marked increase in strongly lignified secondary xylem. With phenylacetic acid and with tryptophane, activity appeared to be greatest in the phloem and endodermis with the development of well-defined vascular bundles, especially with tryptophane. Tetrahydrofurfuryl butyrate induced a response similar in some ways to that resulting from nicotine. With tetrahydrofurfuryl butyrate the cambium was greatly activated in the production of xylem, but there was proliferation of xylem parenchyma and pith at later stages.

The response induced by nicotine in the present experiment most closely resembled that resulting from wounding. In both responses the greatest prolifera-

tion occurred in the meristematic zone originating from the cambium and adjacent undifferentiated tissues. Tumor formation and vascularization were also essentially alike. However, there were differences in the two responses. Nicotine not only considerably augmented the amount of response beyond that which occurred naturally under comparable circumstances but it also induced a response under conditions where the natural response failed to occur. With nicotine, tumor formation proceeded at a predictable rate. With natural wound response a tumor might be formed in 7 days, 21 days, or not at all. These properties of nicotine may be considered significant: nicotine induces a response histologically similar to wound response, and it has the capacity to induce response in greater degree and with greater certainty than in comparable cases of natural wounding.

Summary

1. Nicotine, applied in lanolin to the decapitated second internodes of young Red Kidney bean plants, induced a well-defined response with the formation of a superficial tumor at the stem tip.

2. The tumor was flat-topped with peripheral portions growing downward and closely encircling the stem. The surface was finely tuberculate. There was no evidence of root primordia.

3. Histological characteristics were as follows: (*a*) slight proliferation of cortical parenchyma at cut surface; (*b*) enlargement of endodermal cells with several tangential divisions near the cut surface; (*c*) pericycle crushed or maturing as fibers; (*d*) general proliferation of phloem parenchyma at cut surface; sieve tubes little changed; (*e*) greatest activity in cambium and adjacent undifferentiated cells forming a meristematic zone and resulting in a wedge-shaped area of proliferated tissue; at the apex this tissue continued to proliferate to form a major portion of the tumor; derivatives matured as parenchyma or as a complex of vascular bundles, the largest of which lay along the outer margin of the wedge and were continuous with the vascular tissues of the stem axis; (*f*) in the xylem no activity in the lignified elements; some enlargement of the parenchyma; (*g*) outer portion of rays proliferated as part of the meristematic zone; mainly cell enlargement in inner portions; (*h*) pith inactive and soon disintegrating; (*i*) tumor above the treated surface derived mainly from the derivatives of the meristematic zone; small, peripheral portions from proliferation of the phloem.

4. Observations of wound response were made on decapitated axes of various parts of bean plants. Young decapitated stems responded with weak, irregular tumors or failed to form any callus. Hypocotyls and decapitated older branch axes responded with well-defined callus formation.

5. The histological character of wound response in the bean conformed to that reported for plants in general.

6. The response to nicotine showed greater similarity to wound response than to responses described for various growth-regulating substances.

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EFFECTS OF PRE-STORAGE TREATMENT OF HARDWOOD AND PINE SEEDLINGS WITH α-NAPHTHALENEACETIC ACID

ROGER D. WAY AND T. E. MAKI[1]

Introduction

Although forest tree seedlings usually are planted within a few days after lifting from the nursery bed, storage of stock frequently becomes necessary when, for instance, fall lifting for early spring planting is desirable, or when planting of lifted stock is delayed by such circumstances as a prolonged period of bad weather or a shortage of labor. The principal objective of storage is to maintain dormancy or to hold growth to a minimum without injury to, or deterioration of, the stock. Where refrigerated storage is available, this problem is seldom acute, but where common storage facilities, such as an unrefrigerated cellar or shed, or outdoor heeling-in, must be relied upon, the length of time over which stock can be held without deterioration is definitely limited.

[1] Formerly Research Assistant and Forester, respectively, Northeastern Forest Experiment Station, U.S. Forest Service, Philadelphia, Pa.

In current methods of seedling storage, emphasis has been placed primarily upon proper control of temperature and humidity in the storage chambers (1, 3, 7). Though those factors undoubtedly are of paramount importance, recent advances in the practical application of growth-regulators to various fields of plant culture have spurred hope that these chemicals might also prove helpful for maintaining or prolonging dormancy of seedlings in storage. Recent studies (4, 5, 6) have shown that shoot growth of seedlings, both in the field and in storage, can be inhibited by use of various growth-regulators. Accordingly, several experiments were undertaken at the Beltsville Forest Laboratory[2] to determine the practical utility of growth-regulator treatments for prolonging the time that tree seedlings can be held in common storage. The present paper summarizes the results from tests with naphthaleneacetic acid.

Review of literature

MARTH'S (5) extensive work on roses is the first published study known to the writers that deals with growth-regulator treatments of woody plants in storage. He found that naphthalenemethylacetate, naphthaleneethylacetate, and naphthaleneacetonitrile, applied either as vapor or as spray in proper concentrations, inhibited top growth and conserved the starch reserve of the stock during storage. When outplanted, stock treated prior to storage showed increased root growth, delayed top growth, but, at the end of the season, greater total shoot growth than untreated plants. In the vapor state the most effective concentrations of chemical were 0.3–0.4 mg./cu. ft. of treatment chamber; optimum periods of exposure were about 16 hours at 32°, 4 hours at 40°, or 1 hour at 70° F. Concentrations of vapor above optimum caused injury; concentrations below optimum stimulated shoot development in storage. For use as a spray MARTH recommended a concentration of 0.01% in 0.25% wax emulsion. The most striking effects of the treatments were in preventing deterioration of stock in common storage; however, they also improved the field performance of stock kept in controlled cold storage.

In a series of exploratory experiments begun in the autumn of 1942 at the Beltsville Forest Laboratory, OSTROM (6) obtained highly promising results with hardwood stock held in unrefrigerated storage for late planting. Both vapors and sprays of naphthalenemethylacetate largely prevented development of etiolated shoots of white ash. Dormancy was prolonged for a month or more indoors but for only short periods in an outdoor heeling-in bed. Root initiation in these two types of storage was stimulated both above and below the root collar. Vapors and sprays on white ash held in unrefrigerated storage improved field survival. Treated stock, kept fully dormant in cold storage and planted late, showed a temporary delay in leafing-out and higher survival in the plantation than untreated stock.

The experiments discussed in this paper were in general a continuation and expansion of OSTROM'S work.[3]

Material and methods

The present study was designed to determine the effects of method of applica-

[2] A branch of the Northeastern Forest Experiment Station, near Laurel, Md.

[3] These experiments were conducted in co-operation with the Hormone Project of the Bureau of Plant Industry, Soils and Agricultural Engineering. Special thanks are due Dr. CHARLES L. HAMNER, who prepared the chemicals and assisted in treating and storing the stock.

tion and of concentration of naphthaleneacetic acid as an agent for prolonging dormancy of hardwood and pine seedlings in common storage. It involved two separate schedules of pre-storage treatments and two field plantings. In the first experiment, tests were made of the effects of three methods of applying the growth-regulator (emulsion-dip, vapor, aerosol) on seedlings of black locust (*Robinia pseudoacacia* L.) and loblolly pine (*Pinus taeda* L.). The second experiment tested the effects of a range of concentrations of the growth-regulator, applied in emulsions only, on seedlings of red oak (*Quercus borealis* var. *maxima* Marsh.) and red pine (*P. resinosa* Ait.). Limited quantities of suitable stock necessitated using different species for the two phases of the study. Both plantings were in an infertile sandy loam soil of an abandoned field.

Treatment effects were determined on the basis of seedling response in terms of prolongation of dormancy, survival, vigor, and terminal growth.

Investigation

Experiment I. Method of application

The efficacy of emulsion-dip, vapor, and aerosol methods of growth-regulator applications were compared. For the emulsion-dip method, in which lanolin at 5 gm./l. of water was used as the growth-regulator carrier, preparation consisted first of dissolving naphthaleneacetic acid in melted lanolin in proportions to give concentrations of 100 and 500 mg. of the acid per liter of emulsion. Then the lanolin-acid mixtures were emulsified in the required amounts of water, using monoethanolamine stearate at 10 gm./l. as the emulsifying agent. A third emulsion, prepared as above except that no growth-regulator was added, served as a check of carrier effects. A fourth, with the same concentration of lanolin but emulsified with laundry soap at 10 gm./l., provided a comparison of the effects of emulsifying agents. In treating by the dip method, bundles of seedlings were immersed, tops down, into the liquid to the level of the root collars, were gently agitated to assure complete coverage of all twigs and foliage (of pines), and then were removed and allowed to drain a few minutes before packing for storage.

In the vapor method alcoholic solutions of naphthaleneacetic acid were vaporized on a hot plate in an airtight chamber containing the bundles of seedlings. The latter were exposed to the vapors for 16 hours at about 70° F. at concentrations of 0.4 and 0.6 mg./cu. ft. of chamber space. Control seedlings of the vapor series provided the only untreated group in the test.

Aerosol treatments were applied by the method used by Hamner, Schomer, and Goodhue (2). The aerosol mixture consisted of 1% naphthaleneacetic acid dissolved in 10% cyclohexanone (a mutual solvent for the hormone) and 89% dimethylether (b.p. = 27° C.), which is a liquefied gas and develops 89–90 lb./sq. in. pressure at room temperature and thereby disperses the hormone through a capillary jet, forming an aerosol. The aerosol was released into an airtight chamber containing the bundles of seedlings. As with the vapor treatments, concentrations of the growth-regulator were 0.4 and 0.6 mg./cu. ft. of space, and exposure was for 16 hours at about 70° F. Control seedlings of the aerosol series were exposed to a similar aerosol mixture containing no growth-regulator. For both vapor and aerosol treatments seedling roots were packed in damp sphag-

num wrapped in burlap to prevent drying during the treatment period.

The stock used in these tests was 1-year-old black locust and loblolly pine, lifted, respectively, on April 1 and April 10. For each species, 2000 seedlings were sorted into eighty uniform bundles of twenty-five seedlings each. This provided four replications for each treatment. The complete array of treatments consisted of three methods of application (emulsion-dip, vapor, and aerosol) × three levels of naphthaleneacetic acid (none, medium, and high) × two planting dates (May 12 and May 25), plus one soap-emulsion dip treatment for each planting date, making a total of twenty treatments per species.

Treatments by all three methods were applied on April 11, 12, and 13, 1944. The bundles of seedlings were packed immediately after treatment into bushel baskets with moist sphagnum around the roots and were placed in common storage. The storage house was a half-cellar of masonry walls below ground level and insulated frame construction above ground, with an adjustable ventilator in the roof. Temperatures in the house during storage gradually rose as follows: April 12, 51°; April 22, 53°; May 10, 60°; May 22, 65°; and May 23, 67° F.

The first planting was made on May 12 and 13 and the second on May 25 and 26. The plantation arrangement consisted of four randomized blocks, each split into two plots assigned at random to the two species. The twenty treatments were randomized row-wise within each plot. Rows were 3 feet apart, each containing twenty-five seedlings spaced 2 feet apart. Planting was done by the slit method, using a standard type of tree planting bar.

Results

BLACK LOCUST.—Seedlings of the first planting, when removed from storage on May 10, were still almost completely dormant. Only a few buds had elongated as much as an inch. In contrast, seedlings removed from storage 13 days later (May 23), during which time temperatures in the house ranged between 60° and 67° F., had grown remarkably. Some shoots were 2.5 feet long. Of the several growth-regulator treatments, only the emulsion with 500 mg. of naphthaleneacetic acid per liter had inhibited growth sufficiently to be readily evident upon casual inspection.

The number of shoots over half an inch long was counted on each seedling in the first planting on May 15 and on each seedling in the second planting on May 29. The mean numbers per seedling were, respectively, 1.8 and 3.7; the mean difference of 1.9 is significant at the 1% level. Thus, the extra 13 days in storage had resulted in a marked increase in number as well as in length of shoots. Effects of the treatments on number and total length of shoots on seedlings of the second planting are shown in table 1. Treatment effects on the first planting were generally similar to those on the second planting. Seedling response to the soap emulsion was not different from that produced by the monoethanolamine stearate emulsion.

Inhibiting effects of naphthaleneacetic acid on shoot production were in proportion to its concentration except in the vapor method, where the medium level, for some obscure reason, inhibited shoot production more than the high level. The high-level emulsion was particularly effective. However, the lack of shoots may have been due to outright killing of leaf primordia rather than to true inhibition of shoots.

Survival of seedlings was recorded on July 6 and November 4, 1944, and on May 18, 1945. Since treatment effects were practically identical in the three tallies, only those shown in the last tally are discussed. Neither the method of application nor concentration of the acid caused any significant differences in survival. However, planting dates had a highly significant effect. Seedlings planted May 12 and May 25 showed average survival of 92.4% and 76.4%, respectively. The mean difference of 16.0% is significant at the 1% level.

TABLE 1

INHIBITING EFFECTS OF PRE-STORAGE TREATMENTS WITH NAPHTHALENEACETIC ACID ON SHOOT GROWTH OF BLACK LOCUST SEEDLINGS HELD IN COMMON STORAGE, APRIL 12–MAY 23. SEEDLINGS PLANTED MAY 25, EXAMINED 4 DAYS LATER. BASIS: 100 SEEDLINGS PER MEAN

Method of application	Concentration of acid	Mean no. of shoots over ½ inch long per seedling	Mean total shoot length per seedling (in.)
Lanolin emulsion	0	4.2	29.8
	100 mg./l.	3.9	28.7*
	500 mg./l.	2.6†	23.1†
Vapor	0	4.0	34.5
	0.4 mg./cu. ft.	3.3*	27.5†
	0.6 mg./cu. ft.	3.8	28.4*
Aerosol	0	4.0	34.9
	0.4 mg./cu. ft.	3.7	25.0†
	0.6 mg./cu. ft.	3.7	21.1†

* Inhibiting effects significant at 5% level.
† Inhibiting effects significant at 1% level.

In general, seedling vigor and growth were closely correlated with survival. Vigor was rated according to an arbitrary scale as excellent, good, poor, and very poor. The average proportions of seedlings in excellent vigor on July 6, 1944, were 64.8% for the early and 19.6% for the late planting. The mean difference of 45.2% is significant at the 1% level. Neither methods of application nor levels of naphthaleneacetic acid had any significant effect on over-all seedling vigor. The treatments, however, did influence survival and vigor of the long, etiolated shoots which had developed on seedlings of the May 25 planting during the latter part of the storage period. Though most of these spindly shoots became green and persisted for some time after planting, many of them died later in the season (fig. 1). All growth-regulator treatments and even emulsion alone tended to increase both the percentages of spindly shoots remaining alive and, especially, the percentages displaying excellent vigor (table 2).

FIG. 1.—Effect of naphthaleneacetic acid in preventing development of spindly shoots on black locust seedlings held in common storage for 6 weeks, then planted on May 25, 1944: *left*, untreated seedlings; *right*, seedling tops dipped into 0.5% lanolin emulsion containing 100 mg. of acid per liter. Note dead spindly shoots on untreated seedlings. Photo taken July 26, 1945.

Both shoot elongation and foliage development were, like survival and over-all vigor, unaffected by the growth-regulator treatments but were strongly influenced by date of planting. At the end of the first growing season, total lengths of new growth were significantly greater for the early than for the late planting, despite the in-storage develop-

ment of etiolated shoots up to 2.5 feet long on the late-planted series. Thus, by the end of the first season, the early planting was definitely superior not only in quality but also in amount of new shoot growth. On May 18 of the second season (1945), mean seedling heights were 18.1 inches for the early and 14.5 inches for the later planting. On the same date, 79.2% of the early planted seedlings showed good foliage development (ten or more well-developed leaves per plant) as against 55.6% for the later planting. These differences in height and foliation are statistically significant at the 1% level.

TABLE 2

EFFECT OF PRE-STORAGE TREATMENTS WITH VARYING CONCENTRATIONS AND METHODS OF APPLICATION OF NAPHTHALENEACETIC ACID ON SHOOT GROWTH OF BLACK LOCUST SEEDLINGS HELD IN STORAGE. PLANTED MAY 25, EXAMINED JULY 6. BASIS: 100 SEEDLINGS PER MEAN

Method of application	Concentration of acid	Spindly shoots living (%)	Spindly shoots in excellent vigor (%)
Lanolin emulsion..	0	63.2	30.0*
	100 mg./l.	72.2	32.8*
	500 mg./l.	73.2†	25.2*
Vapor.....	0	58.0	12.5
	0.4 mg./cu. ft.	69.2	24.5*
	0.6 mg./cu. ft.	75.0†	25.0*
Aerosol.....	0	42.2‡	10.8
	0.4 mg./cu. ft.	68.2	30.5*
	0.6 mg./cu. ft.	78.2*	40.0*

* Benefits significant at 1% level.
† Benefits significant at 5% level.
‡ Harmful effects significant at 5% level.

LOBLOLLY PINE.—Measurements were made of the lengths of terminal buds on all loblolly pine seedlings 3 and 4 days after the first and second plantings, respectively. Planting dates had a highly significant effect on bud elongation during storage. Mean terminal bud length in the May 12 planting was 0.83 inch and in the May 25 planting, 0.94 inch. The mean difference of 0.11 inch is significant at the 1% level. The growth-regulator treatments, except for the high-level vapor, did not retard bud elongation in storage in any consistent manner (table 3). The apparent retardation in the aerosol-0 lot may represent chance variation or possibly indicates inhibitory effects of the carrier which were neutralized or counterbalanced in the other two lots by possible stimulating action of the growth-regulator.

TABLE 3

EFFECTS OF PRE-STORAGE TREATMENTS WITH NAPHTHALENEACETIC ACID ON MEAN LENGTH AND VIGOR OF TERMINAL SHOOTS OF LOBLOLLY PINE SEEDLINGS. BASIS: 200 SEEDLINGS PER MEAN (BOTH PLANTING DATES COMBINED)

Method of application	Concentration of acid	Mean terminal bud length (in.)	Seedlings with vigorous terminal buds (%)
Lanolin emulsion..	0	0.91	66.5
	100 mg./l.	.90	41.0*
	500 mg./l.	.89	3.0*
Vapor.....	0	.93	63.0
	0.4 mg./cu. ft.	.88	79.0†
	0.6 mg./cu. ft.	.79‡	78.0†
Aerosol.....	0	.81‡	80.5§
	0.4 mg./cu. ft.	.93	70.0
	0.6 mg./cu. ft.	0.92	64.0

* Harmful effects significant at 1% level.
† Vigor benefits significant at 5% level.
‡ Inhibiting effects significant at 5% level.
§ Vigor benefits significant at 1% level.

The number of seedlings showing vigorous growth of terminal buds a few days after planting was not affected by planting date. The chemical treatments, however, exerted some marked effects on bud vigor (table 3), the acid in emul-

sions being injurious, the other treatments being either neutral or in some degree beneficial.

Survival of loblolly pine seedlings was recorded on May 16, June 29, and November 6, 1944, and on June 19, 1945. Although survival was less for each succeeding tally, treatment relationships underwent no substantial changes; hence, results of only the last observation are discussed.

During the month of May when the plantings were made, the rainfall was nearly 2 inches below, and the mean temperature was 6.0° F. above, the 35-year average. These abnormally dry-and-hot weather conditions resulted in severe mortality of loblolly pine. Winter mortality, though some occurred, was not unduly high.

As with locust, planting dates had a highly significant effect on survival of loblolly pine. Average survival of seedlings planted May 12 was 32.8%; for those planted May 25, 22%. The mean difference of 10.8% is significant at the 1% level.

Results of growth-regulator treatments upon survival are shown graphically in figure 2. The most striking feature is the highly injurious effect of the chemical when applied in emulsions. Results with all other treatments were suggestive of beneficial or injurious tendencies, but only the 0.4-mg. concentration in vapor showed enough difference from controls to be recognized statistically; that difference barely attained significance at the 5% level. A reasonably close parallel is evident between the figures on bud vigor (table 3) and on survival (fig. 2).

By mid-May of the second season, mean height of seedlings in the early planting was significantly greater (at the 1% level) than in the late planting (10.8 *vs.* 9.0 in.). Accurate evaluation of other treatment effects on height was rendered difficult because of the heavy losses and resultant unequal weightings of means. The growth-regulators in emulsions unquestionably had retarded growth; for all other treatments no appreciable differences in growth were apparent.

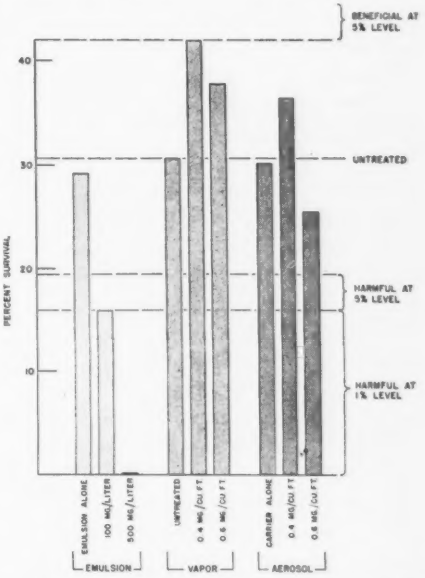

FIG. 2.—Effect of method of application and of concentration of naphthaleneacetic acid on survival of 1-year-old loblolly pine seedlings (planting dates combined). Zero level in vapor series is only untreated group since zero level in other two series represents carriers, aerosol (cyclohexanone and dimethylether) and lanolin emulsion, respectively.

EXPERIMENT II. CONCENTRATION OF CHEMICAL

The primary purpose of this phase of the study was to gain more precise information on the efficacy of different concentrations of naphthaleneacetic acid than could be drawn from the tests in experiment I. Methods of application

were restricted to dipping in lanolin emulsions carrying varying amounts of the growth-regulator. A complete series of treatments was applied to an early lot of seedlings, lifted and treated on March 15 and 16, 1944, and to a late lot lifted and treated on April 22. The stock was 1-year-old eastern red oak and 3-year-old red pine. For each species on each treatment date, twenty uniform bundles of twenty-five seedlings each were assembled.

The emulsions, made as before with 5 gm. of lanolin per liter of water, carried naphthaleneacetic acid in the following concentrations: 0, 10, 20, 40, 80, 160, 320, and 640 mg./l. An additional emulsion carrying 80 mg. of naphthaleneacetic acid per liter was made up, using 5 gm./l. of a commercial wax[4] in place of lanolin; this was for comparison of carrier effects. After the growth-regulator crystals were dissolved in 95% ethyl alcohol, the solution was mixed into the wax emulsion. The commercial wax, though containing an emulsifier, flocculated on contact with the alcohol and, like lanolin, required 10 gm./l. of monoethanolamine stearate to bring about good dispersion. A set of seedlings not treated with growth-regulator served as controls. Thus there were ten treatments in all; these were twice replicated for each of the two species for each date, making eighty treatment units (bundles); at twenty-five per bundle, the total number of seedlings used was 2000.

Treatment consisted of complete immersion of bundles in the emulsions, with gentle agitation to assure full coverage, after which they were removed and allowed to drain. The bundles of seedlings were then packed upright in boxes, with moist sphagnum covering the roots, and were placed in common storage the same day. The following temperatures prevailed in the storage house: March 16, 51°; April 8, 51°; April 12, 51°; April 22, 53°; May 10, 60°; May 22, 65°; and May 23, 67° F.

On May 23 and 24 all seedlings were removed from storage and planted in a manner similar to that described for black locust and loblolly pine. Each of the two blocks in the plantation was divided into two plots to which the species were assigned at random. Within plots the twenty treatment-date combinations were randomized by rows.

In comparing this experiment with experiment I, it should be noted that, although in basic design both entailed two lengths of storage period, that factor was varied at opposite ends: in the second experiment two lifting dates were employed, with planting dates in common; in experiment I two planting dates were employed, with lifting dates in common. This difference provided opportunity to determine whether the major effects of the growth-regulator occurred in the early part or toward the end of the storage period.

Results

Eastern red oak.—On May 31, 8 days after planting, the following records were made of the seedlings: (*a*) number dormant, (*b*) number with some fully developed leaves, and (*c*) the total length of all shoots per seedling. Each of these criteria served as a rough measure of the inhibiting effects of naphthaleneacetic acid concentrations on shoot development.

Treatment date definitely influenced both the numbers of dormant seedlings

[4] The commercial wax was the proprietary product, Dowax, manufactured by the Dow Chemical Company, Midland, Mich. It contains a paraffin wax, bentonite, an emulsifying agent, and possibly other substances. So far as is known, its exact composition has not been published.

and the total shoot lengths. The mean percentage of seedlings dormant in the lot treated March 16 was 7.8; in the April 22 lot, 20.4. Similarly, the mean total lengths of shoots for the two treatment dates were 6.4 and 4.7 inches, respectively. Both differences were significant at the 1% level. These results appear to have been induced by two factors: (*a*) the direct effects of date at which the stock went into storage and (*b*) the increasing sensitivity of the plants to growth-regulator with approach to the date for normal revival of spring growth. Date of treatment had no significant effect on initial leaf development.

The effects of the various concentrations of naphthaleneacetic acid on growth, as indicated 8 days after planting, and on survival 1 year later, are summarized, with treatment dates combined, in table 4. The two highest concentrations definitely delayed break of dormancy and inhibited growth (fig. 3) according to all criteria listed; differences from untreated controls were all highly significant statistically except for leaf development with the 320-mg. concentration. Effects of the intermediate and lower concentrations, except the 0 level, were somewhat erratic, with inhibitory tendencies generally showing in the data on leaf and shoot development but not in the data on dormancy. In fact, the dormancy data alone suggest stimulatory effects from the lower concentrations. Results with the lanolin-80 treatment were particularly suggestive of stimulation.

Survival 1 year after planting was somewhat better for all naphthaleneacetic acid treatments than for untreated controls, except for the 640-mg. concentration (table 4); for four treatments the increases in survival were significant. In view of their originally strong inhibiting effect, it is of interest that the two highest concentrations of the acid caused no appreciable mortality. Date of treatment had no significant effect on survival.

Treatment dates, however, did affect seedling vigor, at least in so far as vigor was expressed by foliage development.

FIG. 3.—Effect of naphthaleneacetic acid on 1-year-old eastern red oak seedlings held in common storage from April 22 to May 23, 1944: *left*, untreated seedlings; *right*, entire seedlings dipped before storage into 0.5% lanolin emulsion containing 640 mg. of acid per liter. Seedlings completely dormant when placed in storage. Note proliferation of new roots from treated stock. Photo taken May 23, 1944.

Seven weeks after planting, the number of vigorous leaves per seedling was significantly greater (at the 1% level) for the April 22 treatment than for the March 16 series, and 52 weeks after planting the same highly significant difference still prevailed. Thus the greater degree of inhibition in storage associated with the later treatment presaged a higher degree of vigor after planting that carried over at least into the second year. This accords with the generally accepted

principle that for best results seedlings should be dormant, or essentially so, when transplanted. The interesting point is that the difference in degree of dormancy associated with the two treatment dates resulted in persisting differences in vigor of growth, even though not registering in terms of survival. It lends emphasis to the frequently ignored fact that differences between treatments in planting experiments often cannot be evaluated adequately by merely recording survival or mortality.

TABLE 4

EFFECT OF PRE-STORAGE TREATMENTS WITH VARIOUS CONCENTRATIONS OF NAPHTHALENEACETIC ACID ON SHOOT DEVELOPMENT AND SURVIVAL AFTER PLANTING OF EASTERN RED OAK SEEDLINGS. BASIS: 100 SEEDLINGS PER MEAN (MARCH 15 AND APRIL 22 TREATMENTS COMBINED)

TYPE OF EMULSION	CONCENTRATION OF NAPHTHALENE-ACETIC ACID (MG./L.)	MAY 31, 1944			MAY 30, 1945, SURVIVAL (%)
		Completely dormant seedlings (%)	Seedlings with some fully developed leaves (%)	Mean total length of seedling shoots (in.)	
None	Untreated control	12.0	51.0	7.1	26.0
Lanolin	0	5.0	63.0	7.6	30.8
Lanolin	10	4.0	37.0	6.4	38.8
Lanolin	20	6.0	50.0	6.3	52.8*
Lanolin	40	5.0	46.0	6.1	48.0*
Lanolin	80	3.0†	67.0	6.2	42.0
Comm. wax	80	8.0	42.0	5.9‡	54.0*
Lanolin	160	10.0	41.0	5.2§	56.0*
Lanolin	320	31.0§	45.0	3.7§	38.0
Lanolin	640	57.0§	14.0§	1.4§	24.8

* Survival benefits significant at 5% level.
† Growth stimulation significant at 5% level.
‡ Growth inhibition significant at 5% level.
§ Growth inhibition significant at 1% level.

RED PINE.—Records were limited to a single tally of survival on June 30, 1944, because heavy mortality (untreated controls 59.2%) rendered further observations useless.

Treatment dates had a highly significant effect. The survival of seedlings treated on March 16 was 18.6%; of those treated on April 22, 7.6%. Thus, in final results, the effects of date of treatment were opposite for red pine and red oak. However, this species difference apparently is merely a reflection of differing degrees of sensitivity and not a basic difference in nature of the reaction to the chemical. The difference in sensitivity may, at least in part, be a matter of proximity to the date for normal breaking of dormancy rather than inherent species characteristics (i.e., red pine normally renews growth early in the spring whereas the oaks are relatively late). With the oak, treatment resulted in a beneficial degree of growth inhibition in storage and more vigorous growth after planting; with the pine, buds were elongating by the later date and in such a state of tenderness that effects of the chemical exceeded mere inhibition to the point of injury.

The effects of all naphthaleneacetic

acid treatments, except at 20 mg./l., were distinctly harmful to red pine, the three highest concentrations causing complete mortality.

Discussion

The principal objective in treating forest planting stock with growth-regulating substances before or in storage is to facilitate holding the stock dormant and without deterioration. Delaying the break of dormancy is a requisite of successful storage because, with routine forest planting practices, most species will not satisfactorily survive transplanting unless the stock is either fully dormant or but little advanced beyond that state. Though survival is the first criterion of success in planting, subsequent growth and vigor also are important considerations that may be influenced by storage treatments or conditions.

In this study involving only unrefrigerated storage, both storage periods and naphthaleneacetic acid treatments affected seedling survival and growth, but the effects of storage periods were generally more pronounced. Black locust and loblolly pine deteriorated considerably in storage, especially during the last 2 weeks of a storage period extending into the warm weather of late May. Inasmuch as storage periods for these two species were varied only through different termination dates (not beginning dates), the tests with them unfortunately involved some confounding of effects of date of planting with the deteriorative effects of storage. However, even freshly lifted locust obviously could not have survived much better than the locust stock of the shorter storage period, since the latter averaged over 90% survival in an abnormally dry summer. The formation of large numbers of spindly shoots in the late-planted lot, and its much poorer survival (76%) and growth must be charged, therefore, largely against deterioration in storage. Freshly lifted loblolly pine from the same lot as used in this study, planted in an adjacent block on April 14, showed an average survival of 54% at the end of the first growing season. Corresponding values for the stored stock in the present test were 50 and 24%, respectively, for the shorter and longer periods. Thus practically all the deterioration in loblolly pine, as in locust, apparently occurred during the last 2 weeks of storage, when temperatures in the storage room ranged between 60° and 67° F.

Effect of length of storage period can be evaluated somewhat better for red pine and red oak, since with these species there was no confounding with planting date. Particularly for oak, growth in storage tended to be less and subsequent field performance better with shorter than with longer storage. However, inasmuch as seedlings of these species were treated on the different dates on which they were put into storage, some confounding of effects again was introduced between storage periods and condition of stock when treated. In general, the latter factor seems to have been more important than length of storage. Both species had become more sensitive to the acid by the time of the later treatments on April 22. The pine buds were swelling and were so tender that the seedlings suffered considerable injury, resulting in higher mortality after planting than was shown in the series treated earlier. The red oak, on the other hand, had by the later date become more sensitive only to the extent that the treatments were more effective in inhibiting subsequent growth in storage, and, in consequence, displayed greater vigor after planting in the field. The difference in

reaction to treatment was not great enough to affect survival.

Response to naphthaleneacetic acid varied with species, ranging from definite injury to significant benefits. The pines were generally more susceptible to injury than the hardwoods. Practically all emulsion-dip treatments, in particular, were injurious to both loblolly and red pines. The fact that, with the pines, foliage as well as woody portions were treated probably accounts in part for the differences in severity of effects between the two types of stock. These findings are in general agreement with those of OSTROM (6), who found that growth-regulator treatments effectively retarded break of dormancy of white ash held in unrefrigerated storage but lowered the survival of loblolly pine.

MARTH (5) found vapor treatments at 0.3 and 0.4 mg./cu. ft. and sprays at 0.01% (100 mg./l.) to be the most effective concentrations for delaying the break of dormancy in roses. In the present study vapor applications of naphthaleneacetic acid at 0.4 mg./cu. ft. were significantly beneficial and emulsion-dip applications at 100 mg./l. were significantly harmful to loblolly pine. However, red oak, after emulsion-dip applications of 20, 40, 80, and 160 mg./l., showed significantly higher survival than the controls; even with the 640 mg./l. treatment survival was not significantly lowered. With red pine, on the other hand, all emulsion-dips except those carrying 0 and 20 mg./l. of growth-regulator were significantly injurious.

These experiments obviously are not adequate as a basis for making practical prescriptions for nurserymen. They do, however, indicate that, with perfection of details of application technique and timing for different species, pre-storage treatment of stock with growth-regulators has possibilities for practical use. They demonstrate the existence of marked differences in sensitivity to naphthaleneacetic acid and in vulnerability to injury by it, as between the pines and hardwoods tested; these differences between the two taxonomic groups are believed to hold generally for other species. The experiments have reasonably well established the range of concentrations of chemical within which desired effects are most likely to be obtained and have shown something of the relative merits of different methods of application. The emulsion-dip method, for instance, which required the least in special apparatus, apparently is acceptable for treating hardwoods but is unsuited for use with pines. On the other hand, vapor and aerosol methods can be used for both classes of stock and, where facilities are available, probably are to be preferred. They permit more precise control over intensity of treatment than dips; in the latter, intensity is contingent upon the amount of chemical adhering to the plant and the length of time its potency is retained.

The marked stimulation of growth of new roots in red oak during storage (fig. 3)—and similarly of white ash (6)—suggests that the methods here employed for applying growth regulators might prove superior to conventional root-soaking treatments as a pre-planting measure apart from storage. The increased formation of root primordia resulting from treatment should materially improve chances for establishment of newly planted seedlings. Such treatment would necessitate proper timing so that, if storage were involved, planting would be finished before new roots had made enough growth to be susceptible to breakage.

Summary

1. Experiments involving methods of application and various concentrations of naphthaleneacetic acid, applied as pre-storage treatments to prolong dormancy and lessen deterioration of forest planting stock in unrefrigerated storage, were carried out on two hardwood and two pine species. Black locust and loblolly pine were treated with medium and relatively high concentrations of the chemical by three methods: exposure in a closed chamber to vaporized acid, to the acid dispersed in an aerosol mist, and by dipping the plants into lanolin emulsion in which the acid was dispersed. Eastern red oak and red pine were used in a test of eight different concentrations of the acid, applied only by the emulsion-dip method. Following 5–10 weeks' storage, the stock was planted in the field for observations on survival and vigor of growth.

2. Response to the treatments varied widely by species, ranging from significant benefits to distinct injury. The pines were much more vulnerable to injury than the hardwoods.

3. Main effects evident upon removal of the stock from storage were as follows: (*a*) With black locust, shoot elongation in storage was inhibited by both medium and high concentrations of the acid in all methods of application. (*b*) With loblolly pine, shoot elongation was inhibited by the higher concentration of acid applied in vapor form. Effects of other methods and concentrations on shoot elongation were either insignificant or erratic. However, the percentage of seedlings displaying apparently vigorous buds about a week after planting was increased by both levels of vapor treatment, by the lower level of acid in aerosol, and by aerosol alone, whereas the acid-emulsion treatments resulted in definitely lessened bud vigor. (*c*) With eastern red oak, shoot elongation was inhibited by all emulsions carrying 160 mg. or more of acid per liter. Concentrations of 80 mg. or less tended to hasten the break of dormancy slightly but did not result in any greater average shoot length than that displayed by untreated controls. (*d*) With red pine, shoots were not inhibited except as a corollary of injury.

4. Main effects evident after outplanting the stock in the field were: (*a*) With black locust survival was not affected by the chemical treatments, but vigor and survival of spindly shoots developed in storage generally were greater in treated seedlings than in controls. (*b*) With loblolly pine survival was generally improved by treatment with the acid in vapor form and at the lower concentration in aerosol, but only with the lower concentration of vapor was the increase statistically significant. Both acid-emulsion treatments were injurious, resulting in greater mortality than among controls and in retarded growth of survivors. However, the early indications of enhanced bud vigor in the vapor and aerosol series were not evident in greater height growth after planting. (*c*) With red oak survival was generally better for all levels of acid-emulsion treatment except the highest, which fell slightly below controls. For the 20, 40, 80, and 160 mg./l. concentrations of acid, the increase in survival was statistically significant. No differences in vigor due to chemical treatments were apparent. (*d*) With red pine survival was significantly less than for controls with all treatments except 20 mg./l. and lower concentrations of acid, which caused no marked reaction.

5. In general, all species exhibited significantly more growth and greater sur-

vival when planted after shorter (5–6 weeks) than after longer (8–10 weeks) periods of storage. For red oak, by exception, survival was not appreciably affected by storage period, though in vigor of growth this species concurred with the general trend. Even with the most effective treatments, dormancy was not adequately maintained over the longer periods, and some additional deterioration apart from breaking of dormancy apparently occurred.

6. These preliminary experiments indicate that, with additional experience and improvements in treatment techniques, naphthaleneacetic acid and probably other growth-regulator chemicals may have practical value for prolonging the period over which stock can be held in common storage without loss. Present results, however, offer no promise that growth-regulators can supplant refrigeration when storage for more than 1 or 2 months is required.

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VOLUMETRIC STUDIES OF SEEDS, ENDOSPERMS, AND EMBRYOS IN PINUS PONDEROSA DURING EMBRYONIC DIFFERENTIATION

JOHN T. BUCHHOLZ

Introduction

Extensive investigations on the seeds of *Pinus ponderosa* during their development were undertaken in 1942 in order to obtain more accurate information concerning size relationships in the normal schedule of embryonic development. It was believed that a comparison of the embryogeny in large versus small seeds might shed some light on the function of the suspensor, since large seeds would provide more space for the elongation of this member. Such a study could also show whether morphological and anatomical differences exist between developing embryos of seeds of various sizes.

In mature ponderosa pine seeds the embryo represents about 10% of the whole seed volume and approximately 5% of the weight. However, at the stage during which the stem tip and cotyledons are differentiated, the embryo constitutes less than 0.5% of the volume of the seed. Thus the question arises: Does the young embryo begin to differentiate its organs at a size which is more or less constant—

an embryo of fixed volume—or does it differentiate only at a size which is closely related in some proportion to the size of the endosperm and seed?

The pine seed reaches its full size about the time of fertilization, which is more than a month earlier than the stages that are concerned with differentiation of the embryo. The changes involved during hardening of the seed coat do not increase the seed size. The seed in which fertilization failed to take place, or in which the embryo subsequently died, remains fully as large as a normal seed.

The trees which furnished the cones were all open wind-pollinated forest trees. Some ovules contain embryos resulting from self-pollination; others, from cross-pollination. Cross-pollination might represent combinations of diverse biotypes found within the species. On the whole, it is to be expected that the net result from wind pollination would tend to maintain the population in heterozygous condition, for even self-pollination in any particular seed would be likely to combine one of a variety of pairs of gametes in the zygote.

There is the possibility that differences in the embryos among seeds of a given size might be due to heredity. However, differences in heredity may be conditioned by the internal environment within the seed. Thus, if the mean sizes of the embryos are computed for each size class of seed, individual hereditary differences would tend to cancel out, and a close approach to the net effect of the internal environment of the seed could be obtained from the means of sufficiently large frequencies in each class.

Material and methods

The details concerning source, method of collection, and cone size, together with a record of the mean sizes of the embryos of various stages dissected from seeds of various sizes, have been given elsewhere (4). The present study is a continuation of this type of investigation on one of these trees more intensively and with several refinements of method.

In order to obtain embryos in all stages in the seeds of nearly all available sizes, collections from tree 3-38[1] were made separately on August 5, 8, and 12, approximately 6 weeks before seed maturation. From this tree, on which the present data are based, over 1100 seeds were obtained. None of the smallest-sized class of seeds (no. 11) contained embryos. The classes of seed size are given in the earlier paper (4). More than 150 of the seeds that appeared sound had shriveled contents.

In the present investigation, the volumes of the seeds, after sorting by sieves (4), were measured by liquid displacement before dissection. Similarly, following seed-coat removal, the volume of the endosperm was measured for each individual seed by liquid displacement in a capillary tube. Finally, the embryos were dissected out and their volumes were computed from measurements of embryo length and diameter, as defined in earlier papers (3, 4), by the formula $(\pi d^2/4)l$. Embryos of stages VI–VIII (see 3 for definition of stages) were measured for total length and for length of the embryo axes ending in the plumule primordium. The latter could be done by breaking away several cotyledons to expose the shoot tip to view.

The seeds of the most frequent size classes were not all dissected. These groups had been most thoroughly sampled, and continuation of dissection was

[1] The designation 3-38 refers to the tree P-Eld-3-38 of the Institute of Forest Genetics in Placerville, Calif.

deemed unnecessary. However, the 632 embryos which were dissected out constitute a population three times as large as any of the samples previously obtained. Embryos in all important stages from seeds of varying sizes were thus provided. Upon dissection, the stage of development of the largest embryo in each seed was noted, as well as that of the second largest, and the number of cotyledons was recorded.

Investigation

The duration of embryonic selection in the pine extends for more than a month. During this period of development the only change that may be recognized in the embryo complex is a very gradual increase in the size of the embryos, especially of the largest one, which usually assumes a dominant position at the forward end of the group.

The embryos are only slightly developed during this time of competition. There is a polarity indicated by the differentiation between the suspensor system and the meristematic cylinder borne on the end of the suspensor in each embryonic unit. The suspensors are derived from the elongation of successive layers of cells cut off previously by the embryos. Thus each embryo spins out its own suspensor which continually subtracts cells from the increasing volume of meristematic cells of the embryonic cylinder. The embryo adds unicellular elements (embryonal tubes) to the suspensor at first. Later the multicellular secondary suspensor (of numerous parallel embryonal tubes) is elaborated. Thus the struggle between embryos becomes one of competition in the rate of growth due (*a*) to elongation of suspensor tissue and (*b*) to meristematic increase of cells in the respective embryos.

About 2 weeks after fertilization an embryo emerges from the complex in a dominant position. Its cylinder of meristem enlarges gradually despite rapid development of its suspensor. The twists and coils appearing in the suspensor complex dislocate the orientation of embryos in secondary positions. Snags or spurs of embryonal tubes belonging to the suspensor of the largest embryo ensnare smaller embryos and push them back toward the micropyle. In this way, about 5 weeks after fertilization, the largest embryo—usually in terminal position in the complex—begins its differentiation (fig. 1).

The embryos that are left behind in embryonic selection within each seed usually fail to develop beyond this stage. Most of them remain much less developed. The data of this study indicate that the second largest embryo in a seed rarely exceeds stage II in size, even when it persists to seed maturity.

Differentiation of embryo

After the period of embryonic selection, differentiation of the dominant embryo progresses with considerable rapidity. Although internal differentiation occurs earlier, there is no outward criterion until stage III is reached, when the primordium of the shoot tip appears (figs. 1, 2). The primordium is a special meristematic area at the tip of the larger embryonic cylinder. In dissected embryos the plumule primordium is a very conspicuous area which appears more translucent, probably owing to the character of the vacuoles and other cell contents and to the lack of intercellular air spaces within this tissue.

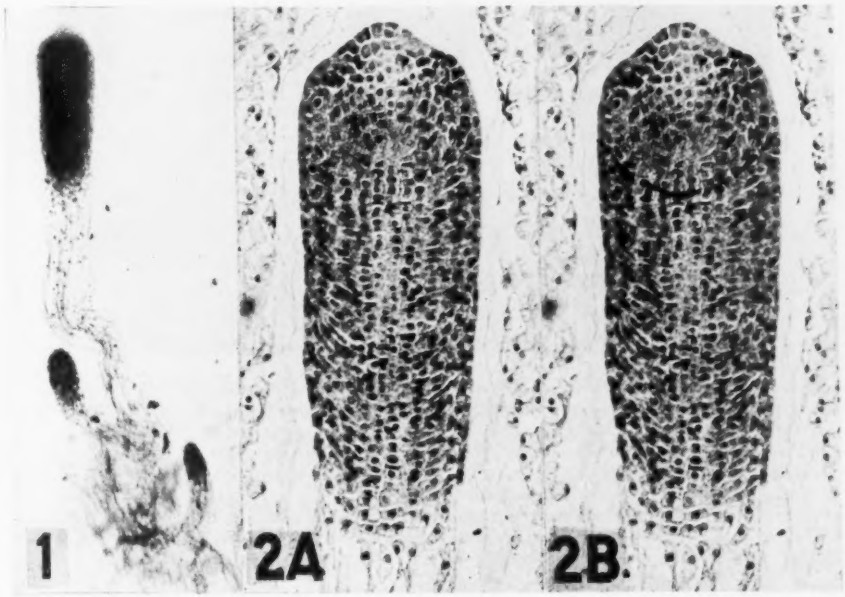
The embryos in seeds within the same cone or other cones of a given tree will not be found in the same stage of development 5 weeks after fertilization. Those whose growth has been most rapid may

be in an advanced stage of development, with their cotyledons already formed and well elongated, while others may still fail to show the shoot-tip primordium. Chronologically, they are of the same age, since fertilization takes place in all ovules of a cone within a few days. It was necessary, therefore, to define a series of morphological growth stages for the embryo which were designated by the Roman numerals I–VIII. These stages were described elsewhere (**3**). It may be recalled here that the origin of the plumule apex occurs in stage III (see figs. 1, 2) and that the cotyledonary primordia arise in stage IV (figs. 3, 4). Figures 5 and 6 show stage V.

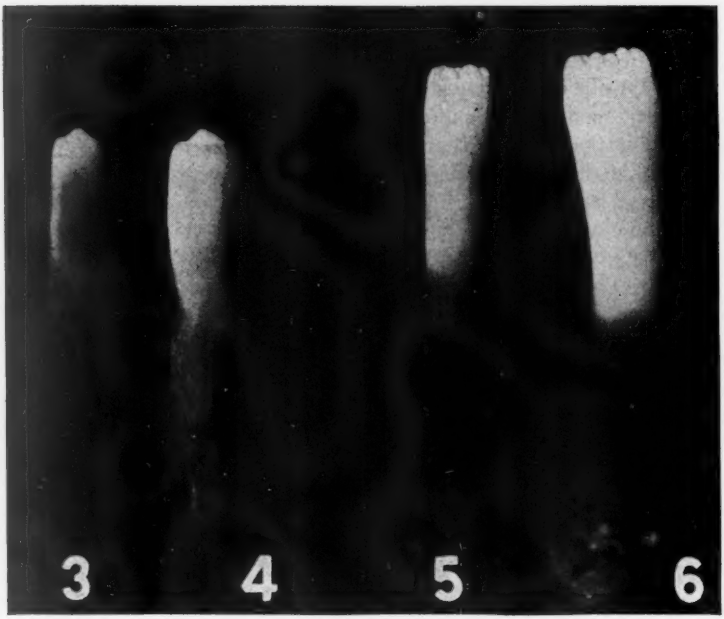
Duration of stages

The time intervals between stages have been calculated in the embryos of a few trees of *P. ponderosa*. The rapidity of growth was quite marked. Two collections were made 4 days apart from tree 3-14 and were used in an analysis that would indicate the duration of the stages. Seventy-eight per cent of the embryos were in stages I–IV on August 1 and only 30% on August 5; 22% were in stages V–VIII on August 1 and 70% on August 5. Table 1 indicates the frequencies and percentages of embryos in different stages on these two dates.

With the aid of table 2, it is possible, using the percentages given in table 1, to



Figs. 1–2.—Fig. 1, dissected embryonic system of *Pinus banksiana*, showing dominant embryo advanced to stage III while other embryo units are being suppressed. ×25. Fig. 2*A*, *B*, longitudinal sections of pine embryo (*Pinus banksiana*) in stage III, at the time when shoot tip is differentiated. Note internal differentiation of small spherical meristematic mass (marked in 2*B*), from which will develop shoot and root of mature embryo. Larger portion below is root cap and suspensor. ×80.



FIGS. 3–6.—Dissected embryos of *Pinus ponderosa*. ×15. Fig. 3, stage IV from no. 13 seed with endosperm volume of 20 cu. mm. Relatively small plumule primordium surrounded by eight cotyledons. Fig. 4, stage IV from large no. 18 seed with endosperm volume of 50 cu. mm. Relatively large stem-tip primordium surrounded by primordia of fourteen cotyledons. Larger throughout than embryo in figure 3. Fig. 5, stage V from no. 13 seed with endosperm volume of 24 cu. mm. The seven cotyledons equal in length to plumule primordium. Fig. 6, stage V from no. 17 seed with endosperm volume of 45 cu. mm. Large stem tip now obscured by thirteen cotyledons of approximately its length. Smaller embryos eliminated in embryonic selection may be seen below, where they were pushed back by massive suspensor of large embryo.

TABLE 1

AMOUNTS AND PERCENTAGES OF EMBRYOS IN DIFFERENT STAGES OF DEVELOPMENT ON AUGUST 1 AND 5 (TREE 3-14)

DATE OF COLLECTION	STAGE								TOTAL
	I	II	III	IV	V	VI	VII	VIII	
August 1 (2 cones):									
Frequency	1	13	17	9	9	0	2	0	51
%	*2*	*25*	*33*	*18*	*18*	*0*	*4*	*0*	*100*
August 5 (5 cones):									
Frequency	4	9	7	31	28	44	26	18	167
%	*2*	*5*	*4*	*19*	*17*	*26*	*16*	*11*	*100*
Total embryos (7 cones)	5	22	24	40	27	44	28	18	218

estimate the rate of progress in growth stages passed through during the 4-day interval. Thus, the 2% of the embryos in stage I and one-fifth of the 25% of the embryos in stage II on August 1 remained unchanged on August 5, indicating that these are stages of long duration. The following calculation was used to derive the approximate rate of change in stage per embryo:

TABLE 2

PERCENTAGE CHANGE IN STAGE DURING FOUR-DAY INTERVAL

DATE OF COLLECTION	STAGE I	II	III	IV	V	VI	VII	VIII	TOTAL
AUGUST 1	2	25	33	18	18	0	4	0	100
ESTIMATED SHIFTS THROUGH 3, 2, 1, OR 0 STAGES	2	5	4	16	17	13	5	7	
				3		13	11	4	
AUGUST 5	2	5	4	19	17	26	16	11	100

Growth changes through three stages:

$7 + 5 + 13$ = 25 embryos × 3 = 75 changes in stage.

Growth changes through two stages:

$11 + 13 + 17 + 16$ = 57 embryos × 2 = 114 changes in stage.

Growth changes through one stage:

$4 + 3 + 4$ = 11 embryos × 1 = 11 changes in stage.

No change in stage:

$2 + 5$ = 7 embryos × 0 = 0 change in stage.

Total 100 embryos with 200 changes in stage in 4 days,

or 100 embryos with 50 changes in stage in 1 day,

or ½ change of stage per embryo per day.

It is possible that the stages are passed through more rapidly than the above rate. The seeds of another tree, 3-57, were collected at an interval of only 1 day. This tree had relatively fewer embryos in stages I and II; hence the rate of change of stage was more rapid, being more nearly two-thirds stage per day. The seeds of tree 3-38 were collected on August 5, 8, and 12, but the seeds of August 5 were not all kept accurately separated from those collected on August 8. However, it was possible to use the data by adding together the seeds of August 5 and 8 and comparing them with the collection of August 12, some of which were dissected on August 13. The mean time between collections was 6–6½ days, giving a rate of 0.47 stage per day for a 6½-day period and 0.52 stage per day for a 6-day interval.

The large number of seeds from tree 3-38 made it possible to compare the intervals between embryonic stages separately in seeds of different sizes. It was found that the rate of change in stage was 0.57 stage per day for no. 17 seeds, 0.54 stage per day for no. 16 seeds, 0.62 stage per day for no. 15 seeds, 0.42 stage per day for no. 14 seeds, and only 0.35 stage per day for no. 13 seeds. Thus it appears that the embryos of seeds of

average size (no. 15) grow most rapidly and that the embryos in the smallest seeds develop much more slowly. The slower rate of growth in the largest seeds might be due to the greater expenditure of tissue in suspensor formation and to the fact that the embryos must grow larger before differentiation. It should be noted that embryos of the largest seeds differentiate only after the embryonic cylinder has become considerably larger than in those of average size (compare fig. 3 with fig. 4 and fig. 5 with fig. 6).

There are evidently some variations in the intervals between embryonic stages, but an interval of 2 days (at the rate of 0.5 stage per day) is used for convenience in discussion in the following pages.

Embryo size in relation to size of endosperm and seed

Table 3 shows results of measurements made on embryos, endosperm, and seeds and includes all stages that were dissected from twelve cones of tree 3-38. It is clearly indicated that the embryos of large seeds, at the time of stem-tip and cotyledon differentiation, are much larger than those of small seeds.

In order to test the volumetric relations of embryos in each stage against the

TABLE 3

EMBRYO LENGTH AND DIAMETER IN RELATION TO SIZE OF SEED, VOLUME OF ENDOSPERM, AND STAGE OF DEVELOPMENT FOR 632 SEEDS*

Seed class	Endosperm (vol. cc.)	Stage I			Stage II			Stage III			Stage IV			Stage V		
		f	*d*	*l*	*f*	*d*	*l*	*f*	*d*	*l*	*f*	*d*	*l*	*f*	*d*	*l*
12	0.018	2	206	270	2	352	614	4	308	738	4	582	1444	1	775	1906
13	.023	11	232	333	9	377	599	7	457	911	16	624	1421	6	768	1948
14	.029	10	252	378	8	375	697	22	466	958	30	629	1463	6	770	1891
15	.035	19	249	346	23	396	634	27	497	983	54	667	1471	17	838	2042
16	.040	9	245	388	10	403	654	14	536	1017	19	682	1480	22	851	2161
17	.046	8	251	346	15	431	699	13	528	1089	16	715	1632	14	922	2132
18	0.052	...	...	...	...	...	...	4	620	1250	5	746	1659	1	946	2294
Mean total	.....	59	245	353	67	400	655	91	498	993	144	662	1492	67	847	2080

TABLE 3—*Continued*

Seed class	Endosperm (vol. cc.)	Stage VI				Stage VII				Stage VIII			
		f	*d*	*tot.l*	*ax.l*	*f*	*d*	*tot.l*	*ax.l*	*f*	*d*	*tot.l*	*ax.l*
13	0.023	4	798	2352	2239	...	...	...	...	...	...	...	...
14	.029	2	876	2596	2426	...	...	...	...	...	...	...	...
15	.035	15	1023	3025	2813	13	1216	4249	3663	22	1446	5322	4232
16	.040	28	1050	3097	2860	11	1230	4394	3768	27	1448	5529	4342
17	.046	18	1114	3221	2969	12	1287	4408	3788	31	1548	5535	4409
18	0.052	2	1023	3054	2806	11	1316	4544	4041	8	1629	5725	4478
Mean total	.....	69	1040	3055	2827	47	1282	4391	3808	88	1475	5444	4370

* Diameter of embryo is *d*, frequency is *f*; *l* is length in first five stages. In last three stages, *ax.l* is length of axis alone, and *tot.l* is over-all length including cotyledons; lengths and diameters are given in microns.

TABLE 4*

RELATIONSHIP OF EMBRYO SIZE (VOLUME) TO SEED VOLUME AND ENDOSPERM VOLUME DURING EXTERNAL DIFFERENTIATION†

SEED SIZE		STAGE III					STAGE IV					STAGE V					STAGE VI					END. VOL. COMPUTED AS 36% OF SEED VOL.
Class	Vol. cu. mm.	*f*	Vol. end. cu. mm.	Vol. emb. cu. mm.	Emb. Seed (%)	Emb. End. (%)	*f*	Vol. end. cu. mm.	Vol. emb. cu. mm.	Emb. Seed (%)	Emb. End. (%)	*f*	Vol. end. cu. mm.	Vol. emb. cu. mm.	Emb. Seed (%)	Emb. End. (%)	*f*	Vol. end. cu. mm.	Vol. emb. cu. mm.	Emb. Seed (%)	Emb. End. (%)	
12.....	50	4	16	0.092	0.18	0.57	4	21	0.384	0.78	1.83	1	19	0.899	1.80	4.73	....	....	.....	.....	.....	18
13.....	64	7	27	.149	.23	.55	16	24	.437	.68	1.82	6	24	0.902	1.41	3.76	4	24	1.176	1.84	4.89	23
14.....	82	22	29	.163	.20	.56	30	27	.455	.56	1.68	6	28	0.882	1.08	3.12	2	28	1.565	1.91	5.58	29
15.....	97	27	36	.176	.18	.49	54	33	.514	.53	1.56	17	34	1.126	1.17	3.30	15	35	2.486	2.57	7.11	35
16.....	113	14	29	.229	.20	.59	19	36	.451	.40	1.25	22	35	1.229	1.09	3.63	28	35	2.682	2.38	7.65	41
17.....	126	13	47	.238	.19	.51	16	43	.655	.52	1.52	14	42	1.423	1.13	3.48	18	44	3.048	2.42	6.90	45
18.....	140	4	49	0.377	0.27	0.77	5	48	0.596	0.42	1.20	1	50	1.612	1.15	3.22	2	50	2.510	1.79	5.00	50
Mean.....		7	....	0.203	0.21	0.58	7	....	0.499	0.56	1.55	7	....	1.152	1.28	3.61	6	....	2.244	2.17	6.21	.....

* In obtaining volumes for this table by liquid displacement, an experimental error up to 8 cu. mm. may have been introduced by the estimation of meniscus level between wide graduations. Thus there was considerable fluctuation in the ratio of endosperm volume to seed volume. The mean value obtained for the endosperm volume was 36% of the seed volume. This figure was used to establish a computed volume of the endosperm in each size class—the column at the extreme right.

† Frequency is designated by *f*. Embryo, endosperm, and volume are abbreviated. See text for further explanation.

volumes of endosperms and seeds, the mean volumes of the embryo were computed for the four stages which involve stem-tip and cotyledon formation. These are shown in table 4. The best test that could be devised which might show a constancy of volumetric relations was that of computing the ratio of volume of embryo to volumes of endosperm and of seed. These values, in percentages, are entered in the appropriate columns of table 4.

The embryos have a closely constant ratio to the size of seed and of endosperm during the stages in which shoot tip (stage III) and cotyledons (stage IV) first appear and begin to elongate. During stage III the embryo constitutes only 0.2% of the volume of the seed and 0.6% of the volume of the endosperm. Errors introduced by use of the formula for a cylinder, as described above, could be partly overcome by making corrections for the domed curvature of the shoot tip, for irregularities due to cotyledons, and for taper below, but these corrections would be nearly constant and would not change the relative values.

It may be concluded that the volume of the embryo at the time of differentiation of its shoot tip (stage III) is closely related to the volume of the endosperm; it is also related to the volume of the seed. The larger embryos have larger shoot-tip primordia which seem to be proportional to the size of the embryo. The volume of the embryo at the time of initiation of cotyledonary primordia (stage IV) is also closely related to the volumes of the endosperm and seed. Although at this stage the ponderosa pine embryo has about two and a half times the volume it possessed at shoot-tip initiation, it still amounts to less than two-thirds of 1% of the volume of the seed and less than 2% of the endosperm volume.

TABLE 5

FREQUENCY OF COTYLEDON NUMBER IN EMBRYOS IN RELATION TO SEED SIZE

Seed size	Cotyledon number											
	6	7	8	9	10	11	12	13	14	15	16	Mean
12	.....	.....	2	1	.....	.....	.....	.....	.....	.....	.....	8.3
13	1	1	9	7	5	.....	.....	.....	.....	.....	.....	8.6
14	1	1	7	13	7	8	.....	.....	.....	.....	.....	9.3
15	.....	2	10	19	35	26	15	5	.....	.....	.....	10.2
16	.....	.....	4	15	16	32	34	6	1	.....	.....	10.9
17	.....	.....	1	8	10	28	25	16	2	.....	.....	11.4
18	.....	.....	.....	2	3	5	7	3	3	2	1	12.1

In embryos of all sizes the size of cells and of nuclei as seen in sections at the time of primordium initiation remains essentially the same. This is true in comparable parts of the embryos: root-cap cylinder, shoot-tip primordium, or cotyledonary primordia. A larger volume in the embryo represents a larger number of cells throughout all its parts, with the possible exception of the cotyledonary primordia (see below).

Cotyledon numbers

The frequencies of different cotyledon numbers in seeds of various sizes are presented in table 5. The fact that cotyledon counts from the smallest seeds are based

upon small numbers is unavoidable, since these sizes of seeds were least frequent in the collections. In addition, because of the slower rate of development in these seeds, few were found at the stage of cotyledon initiation.

A correlation between seed size, embryo size, and number of cotyledons developed may be clearly seen from the data. This corroborates the results obtained earlier (4) from a much smaller number of seeds. It might be supposed that there is more room for a large number of cotyledons on the shoulders of large embryos than on small ones. This would be expected if the plumule primordium of a large embryo were larger than in a small embryo and the cotyledonary primordia did not share proportionately in the increase in size of the embryo as a whole.

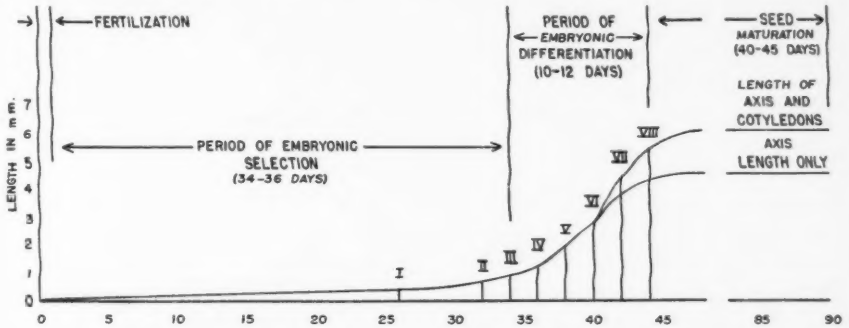


FIG. 7.—Mean growth-curve of embryos in axis length for period of fertilization to maturity of seeds.

GROWTH-CURVE OF EMBRYO

A diagram presenting an over-all picture of the entire program of development from fertilization to maturity (i.e., in growth in length) may be constructed from the data given. Figure 7 represents the average growth-curve for all sizes of embryos of tree 3-38.[2] In this curve the known items are the period from fertilization to stage IV (approximately 35 days), the mean axis lengths of the embryos in various stages, the 2-day mean interval between stages from stage III onward, and the mean axis length of the embryo in a group of mature seeds harvested from the tree 6 weeks later.

It is evident that for a period of about a month following fertilization—the period of embryonic selection—the embryos grow very slowly in diameter and length. After stage III is reached, the dominant embryos enlarge rapidly (figs. 3–6). From stage VIII onward to the maturity of the seed the rate of elongation subsides and is gradually arrested. Between stages VII and VIII the curve falls off sharply only when the axis length is plotted. If the total over-all mean lengths are plotted, the height of the curve continues to increase, owing to the addition of the later-developing cotyledonary meristem. It is clear that during the second portion of the period of development, which involves embryo differentiation, the curve of growth in axis length follows the usual sigmoid pattern.

[2] Since the smaller seeds had no embryos in the later stages of development (table 3), a discrepancy is introduced into these stages on the growth-curve; the basic trend of the curve, however, remains the same.

Discussion

The feature revealed by this and the previous study (4) which is most outstanding is the diversity of stages that may be found among embryos that are collected from a tree on a given date. The embryos are of the same chronological age; they result from fertilizations that take place on the same day or within a few days. However, they are found to diverge as much as 9 or 10 days in terms of the average embryos.

A genetic variation might account for the difference in stage among the dominant embryos of different seeds. The divergence of stages could, therefore, represent a measure of differences in inherent embryonic vigor. An embryo found in stage VIII 35 days after fertilization is 8 days in advance of the mean (then in stage IV). It is a precocious embryo from the standpoint of vigor of growth. An embryo from the same cone which is still in stage I is retarded 8 or more days behind the mean. It has an inferior performance record. The precocious embryo may be rated as growing at the rate of 43/35, or 123% of the average; the backward embryo as growing at the rate of 27/35, or 77% of the average in a given cone or tree. It is possible that these interseminal performance records may hold for the growth rates that are observed in the same individuals after germination of the seeds. Such differences in the vigor of seedlings would be worthy of special attention if they could be realized in cultures of nearly mature embryos. The extremes might seem to add up to the kind of differences in vigor that have been observed between the averages of hybrids and those of normal wind-pollinated progeny (3).

It was thought that this investigation might uncover data which would have a bearing on the function of the suspensor. There was never any doubt as to the importance of this organ as a weapon of competition during embryonic selection, which was pointed out previously (1, 2). Nothing was noted that would serve to alter this conclusion. It would appear that the superior embryo, which always has the largest suspensor, preserves a fine balance between meristematic activity in the embryonic cylinder and suspensor elongation.

When comparisons were made between large and small seeds, it was usually found that the embryos in small seeds were not only smaller in volume per stage but that they were more retarded in the stages which were found. Large seeds yield a greater diversity of stages on a given date, but the most precocious embryos are usually to be found in the medium- and large-sized seeds only. It should not be concluded, however, that all ponderosa pine seeds in the nos. 12–14 group have a slow rate of development. This holds only for trees in which those sizes are smaller than the average. In trees in which no. 13 seeds are the average, that size may have the same or even a more rapid rate of development than the average size (no. 15) in tree 3-38.

The completion of a growth-curve showing the development of the embryo from fertilization into the maturity of the seed has provided an over-all quantitative record of the process of seed development in pines and related conifers. Many features now become more clearly apparent.

The entire embryogeny falls into two periods. The early period, occupying from 4 to 5 weeks in medium and large seeds (longer in the smallest ones) is given over to embryonic selection. Numerous embryos, still more or less undifferentiated, are engaged in an intense in-

ternal "struggle for existence." Usually only a single embryo per seed survives this competition, bounding forth in a spurt of growth and differentiation which assures its success as the selected embryo of the seed. This occupies the second period of development, less than half as long as the first. It is not likely that the same mathematical equation would fit both regions of this curve of growth or that this would be required, since the processes of embryonic development are different in each period.

The first period may be absent in angiosperms which are not generally polyembryonic. If such a period of delay in growth is present in some angiosperms, it may be due to a competition between endosperm and embryo, both of which result from a fertilization process. The writer entertains some doubt that such a relatively prolonged period of slow growth would be evident in angiosperms even where polyembryony is found. It must be remembered that the polyembryony observed in angiosperms does not result in the decisive selection of a single embryo per seed but that usually several embryos are matured within a seed. In pines, in conifers generally, and in all gymnosperms, the usual condition (more than 98%) of polyembryony is terminated decisively in the selection of a single embryo in the seed.

The correlation between seed size and embryo size in ponderosa pine seems significant. The large seeds may offer a better opportunity to the embryo to express a capacity for rapid growth in a short time, while the more crowded condition within a small seed serves to intensify and prolong the period of embryonic selection. Thus, in larger seeds, where there is less crowding among the numerous embryos and where longer suspensors may be produced, embryonic selection may progress more rapidly and come to a decisive conclusion at an earlier date than in the smallest seeds.

However, it is possible that the ratio of size of embryo to size of seed might be explained on a basis other than spatial relations of embryonic development. The nutritional conditions in different regions of the cone may be variable. Hence differences in embryo development could be correlated with nutritional differences occurring in various portions of the cone. It is also possible that seeds vary in their permeability to food materials (according to seed-coat thickness or to the nature of the nucellar tissue) which might be conditioned in association with the factors determining seed size. These and other factors may be involved which are beyond the scope of this study to determine.

Using seed size as a determinant of embryo size, it is possible to speculate on the factors concerned in the establishment of seed size. It is usually assumed that the pollen grains which fall on the micropyle and grow in the ovule furnish the stimulation for ovule development and enlargement. This may be expressed by the liberation of hormones from the pollen grains and tubes. If so, then the stimulus provided may be expected to vary owing to genetic differences among the pollen grains. It may also vary quantitatively, depending upon the number of pollen grains which give rise to active male gametophytes.

There have been workers, on the other hand, who would relate the size of the ovule to other factors, independent of the pollen stimulus. BURLINGAME (5) found that ovule development in *Araucaria* seemed to occur independently of pollen effect. Ovule size may be related to the size of the cone on which it is borne, the position of its own scale with-

in the cone (**6, 8**), and the vigor of the tree. It may also be conditioned by the rate of development of the female gametophyte or by the hormones possibly furnished by that tissue or by the parent sporophyte.

It is generally known that a large seed gives rise to a larger seedling at the time of germination than a small seed. Some foresters have believed that this initial advantage is permanent. Others have contended that the larger seedlings are not necessarily the more vigorous individuals—that the initial advantage in size is only transitory. The latter belief has been borne out by the work of RIGHTER (**7**) and of the author (**3**) in an earlier paper. The discovery in pines that the embryos chalk up their own growth records through a series of easily recognized stages (if observed at appropriate intervals) is an important fact that has been hitherto neglected. It would seem to offer the basis for a new experimental approach to problems of seed selection and improvement.

Summary

1. Data were obtained on the seeds, endosperms, and embryos from cones of previously described trees of *Pinus ponderosa*. Volumetric measurements were made, cotyledons were counted for embryos from different sizes of seeds, and the rate of growth of the embryos was computed.

2. For a period of about 5 weeks there is little embryonic differentiation, the embryos within each seed being engaged in a competitive struggle for survival—a period of embryonic selection.

3. After the dominant embryo has been established, it spurts ahead in rapid growth and differentiates all its organs in a period of 10–12 days. Seed ripening occurs in a few weeks with little change in embryo size.

4. A great diversity of stages is found among embryos collected on a given date. This makes possible a computation of the presumable rate of growth between successive collections of seeds from the same tree.

5. The average rate of growth is about $\frac{1}{2}$ stage per day during embryo differentiation. It is much slower in the smallest seeds.

6. At the time of shoot-tip differentiation and of cotyledon initiation, the volume of the embryo is closely related to the volumes of endosperm and of seed.

7. There exists a correlation among seed size, embryo size, endosperm size, and number of cotyledons in the embryo.

DEPARTMENT OF BOTANY
UNIVERSITY OF ILLINOIS
URBANA, ILLINOIS

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A CYTOLOGICAL STUDY OF UROMYCES ALOËS

M. J. THIRUMALACHAR

Introduction

Uromyces aloës (Cke.) Magn. has been found parasitizing as many as seven species of *Aloe* in Africa (11) and is known to occur in India on *Aloe vera* and *A. spicata*. Only the telial stage was described in the original description of the species; later, by a morphological and cultural study, AJREKAR and TONAPY (1) found the presence of pycnia. They also observed some very interesting features in the germination of the teliospores which invited further study. It was pointed out, for example, that the teliospore on germination puts out a promycelium into which the granular contents of the spore migrate. The germination was stated to be peculiar in that the basidium is two-celled (rarely three-celled) instead of the usual four-celled condition. The upper cell of this two-celled promycelium develops a germ tube and functions directly as the sporidium.

Experience with the cytology of rusts has indicated that the presence of a two-celled basidium is always correlated with some variation in the nuclear sequence. Further, the suppression of the development of sporidia and the consequent assumption by the upper cell of the promycelium of the function of sporidia, would, if confirmed, present us with an interesting variation not usually met with in rusts. This problem was kindly suggested to the writer by Professor S. L. AJREKAR, to whom the author is indebted for many valuable suggestions. On account of the scarcity of specimens, the work could not be undertaken readily. AJREKAR, who first collected the rust near Telegaon, Poona, was unable to collect material again in the same locality. Recently a collection was made by the writer near Kadur, Mysore State, which enabled a detailed cytological study. The present work adds cytological details and thus throws light on the life-cycle; it completely confirms the findings of AJREKAR and TONAPY.

METHODS.—Material was fixed in Karpechenko's modification of Navashin's fluid, and microtome sections 8–10 μ thick were cut and stained either with Heidenhain's iron-alum haematoxylin or with Newton's iodine gentian-violet, counterstained with light green. Teliospores were germinated by the method suggested by the writer (**26**). Gentian-violet and light-green combinations were used in most cases.

Observations

The rust is foliicolous and amphigenous, with a yellowish ground color interspersed with concentrically arranged erumpent sori containing chocolate-brown powdery spores. The spots often coalesce with one another and thus occupy a major portion of the leaf surface.

SPORE-FORMS

PYCNIA.—Pycnial initials are formed by the grouping of hyphae beneath the epidermis and are developed following sporidial infections. They are minute, orange-yellow, and concentrically grouped. Mature pycnia (fig. 1) are flask-shaped, flattened at the apex, and open out by a small pore formed by the rupture of the epidermis. There are no traces of any

ostiolar flexuous hyphae projecting beyond the pore. The pycniospores are extruded and collect in a tiny droplet of secreted nectar on the outer surface. The pycnial initials, the mycelium bearing the pycnia, and the pycniospores are all uninucleate.

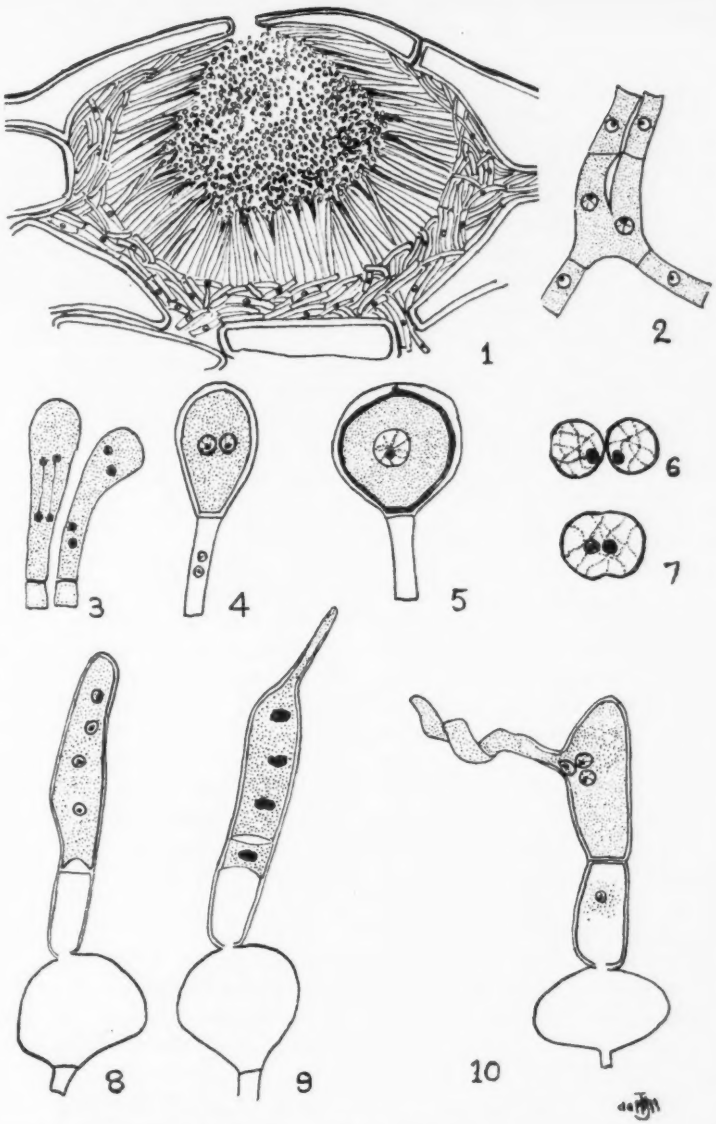
TELIA.—The telia are formed soon after the development of the pycnia. Macroscopically the telial initials appear on the surface of the infection patch as yellowish flattened pustules. These are sometimes formed directly after sporidial infections in an abbreviated cycle. Even after completion of the development of telia, the mycelium bordering the infection patch spreads toward the base of the leaf, developing new pycnia and telia. Since it does not reach the shoot growing-point of the host (which is at the base of the radical leaves), the rust does not become systemic.

Sections through a young telium show a plectenchymatic mass of hyphae grouped beneath the leaf epidermis. Cells of the hyphae are all uninucleate. Toward the center of the telium primordium and generally extending toward its periphery, cell fusions between neighboring cells take place. These are effected by dissolution of the separating wall, either partially or completely (fig. 2). Such types of cell fusions have been described in *Phragmidium speciosum* (7), in *Phragmotelium mysorensis* (27), and in *Thekopsora hydrangeae* (22) within the *aecial primordia*.

Following production of the fusion cells, the other plectenchyma cells begin to disintegrate. The binucleate fusion cells elongate in a vertical direction, and each cuts off a stalk cell and a teliospore (figs. 3, 4). The two nuclei within the pedicel remain small and inconspicuous and finally degenerate prior to the maturity of the teliospore. The wall of the spore thickens and turns yellowish-brown in color. The two nuclei fuse and form a single large fusion nucleus (fig. 5). Prior to fusion the two nuclei develop a characteristic structure found during the prophase of the mitotic division (figs. 6, 7). This has been referred to as the nucleus of the expanded type by SAVILE (25). In the early stages following nuclear fusion the two nucleoli remain distinctly separate but finally fuse. The mature spore is thick-walled, with a single large fusion nucleus (fig. 5) and an apical germ pore, which in some cases is laterally situated. The pedicels, which are very long—up to 130 μ—are fragile and break up just beneath the point of the hilum. Consequently, the mature teliospores are dispersed very much like the urediniospores of other rusts and impart a dusty, powdery appearance to the sorus.

BASIDIA.—The teliospores germinate readily without a period of rest being necessary. Prior to germination the spore coats slightly swell in water, thus revealing the distended germ pore. The promycelium emerges as a small papilla, gradually elongating in length and becoming cylindrical. The granular contents of the teliospore along with the fusion nucleus migrate into the promycelium. Unlike some of the rust species studied by SAVILE (25), the prophase stages of the nuclear divisions do not take place within the teliospore itself. As a result of two successive divisions of the fusion nucleus, four free nuclei are derived and lie in a linear row (fig. 8). Further development of the basidium is uniformly consistent in a majority of the cases, and only a few variations have been observed.

In the normal type of development, after the formation of four free nuclei in the promycelium, the lowermost nucleus is separated off by a septum (fig. 9)



FIGS. 1–10.—Fig. 1, pycnium, ×400. Fig. 2, cell fusion in telial primordium, ×1800. Fig. 3, teliospore initials, ×800. Fig. 4, young teliospore, ×800. Fig. 5, mature teliospore, ×800. Figs. 6, 7, stages in formation of fusion nucleus, ×1800. Fig. 8, germinating teliospore with linear row of four nuclei, ×800. Fig. 9, separation of lower promycelial cell, ×800. Fig. 10, development of infection branch, ×800.

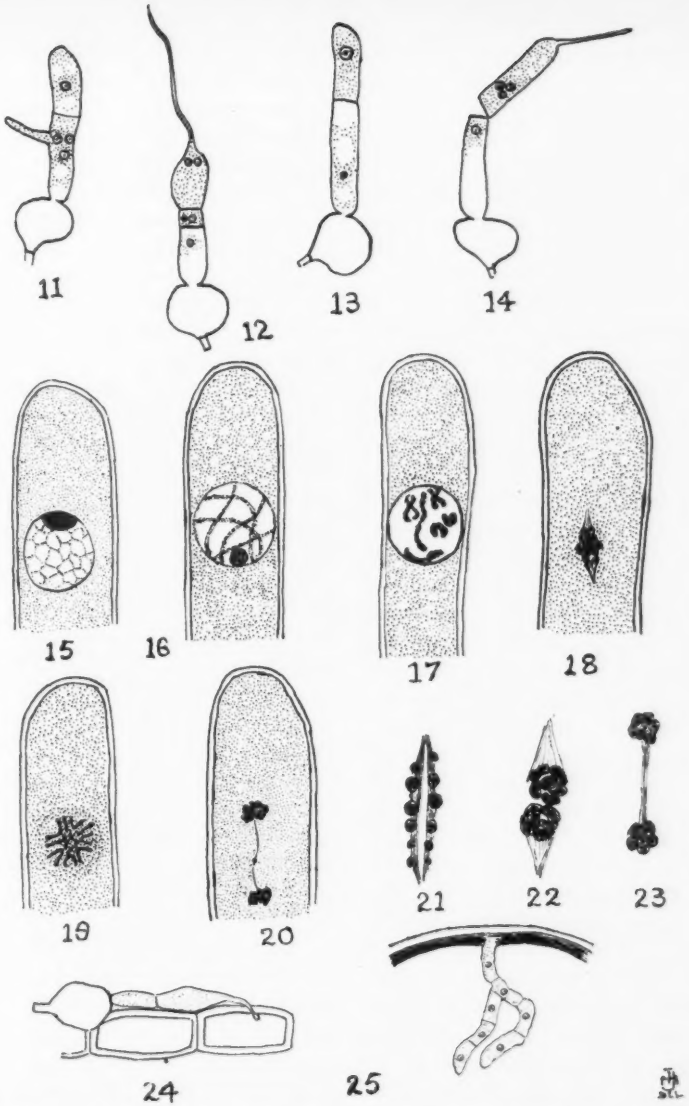
which appears to be laid out by cleavage furrows. This is facilitated by the differentiation of small vacuoles which coalesce with one another. The two-celled promycelium thus produced has an upper three-nucleate cell and a lower uninucleate cell. The apical portion of the upper cell very soon elongates into a whiplike process into which the three nuclei migrate along with the cytoplasmic contents. This whiplike structure, although usually terminal, often develops in a lateral position (fig. 10). In the lower cell the nucleus remains inactive and in later stages degenerates. The cell contents become highly vacuolated and in some instances undergo shrinkage.

In one of the observed variations the entire process of development usually found in the upper cell of the promycelium is restricted to the lower cell (fig. 11); i.e., the upper cell is uninucleate and sterile, whereas the lower one is three-nucleate and develops the infection hypha. In another instance, after the linear row of four nuclei was organized in the promycelium, two septa were laid separating three cells. The upper cell was binucleate. The middle cell showed some activity in having a single nuclear division and becoming binucleate (fig. 12), but very soon all the nuclei within the two lower cells degenerated, leaving only the upper fertile cell. In such cases the promycelium appears to be three-celled, but only one of the cells is fertile. The three-celled promycelia observed by AJREKAR and TONAPY might be of similar nature. In still another type of variation, after the first meiotic division and the separation of the two daughter nuclei, the lower one degenerates and the upper one, by further division, forms a binucleate promycelial cell (fig. 13).

The promycelium in *U. aloës* is thus usually two-celled, with one fertile cell and one sterile cell, or three-celled, owing to the presence of two sterile cells. The fertile cell develops a whiplike structure which, during infection, directly penetrates into the host and is therefore of the nature of an infection branch. In other rusts the cells of the promycelium may develop a whiplike branch in place of a sporidium when excessive moisture conditions prevail during teliospore germination. Such abnormal types of teliospore germination are common among partly submerged spores with limited oxygen supply. In *U. aloës*, however, even in a seemingly suitable environment, the development of basidiospores has never been observed. It seems certain that they are not formed. The promycelial cell has assumed the function of the sporidium and develops the infection branch. The rounding-up of promycelial cells (fig. 14) which behave like sporidia has been recorded in species of the genera *Chrysocelis* Diet. and *Acervulopsora* Thirumalachar. AJREKAR and TONAPY compared it with the genus *Barclayella* Diet. (=*Chrysomyxa*), in which, however, the rounding-up of promycelial cells has been ascribed to abnormal conditions prevailing during germination.

MEIOSIS

The following cytological details were observed for the meiotic divisions of the fusion nucleus in the promycelium. The fusion nucleus shows a finely staining reticulum of chromatin with a nucleolus which, in most of the cases observed, is situated on the side of the nucleus toward the tip of the promycelium (fig. 15). The nucleolus stains deeply with gentian-violet and haematoxylin. The first indication of the beginning of the prophase is the condensation of the chromatin into long, narrow, sinuous strands. These



FIGS. 11–25.—Figs. 11–13, variation in type of teliospore germination, ×400. Fig. 14, upper promycelial cell separating, ×400. Figs. 15–23, meiosis. Fig. 15, resting nucleus, ×1800. Fig. 16, zygonema stage in promycelium, ×1800. Fig. 17, diakinesis, ×1800. Fig. 18, first metaphase, ×1800. Fig. 19, stage before organization of spindle, ×1800. Fig. 20, anaphase, ×1800. Fig. 21, late metaphase, ×3600. Fig. 22, clumping of chromosomes at either side of equatorial plate, ×3600. Fig. 23, anaphase, ×3600. Fig. 24, penetration of infection hypha through epidermis, ×400. Fig. 25, septate mycelium composed of uninucleate cells after infection, ×800.

are distributed within the nucleus without any definite arrangement and constitute the leptotene stage. Many nuclei have been observed in the zygonema stage showing the pairing of chromosomes (fig. 16). Very soon after this pairing, the chromosomes contract abruptly in length and become short and stumpy. Six pairs of chromosomes have been counted in most of the preparations showing diakinesis (fig. 17). The nuclear membrane disappears at this stage, and the first indication of the organization of the spindle is observed. The nucleolus disappears completely just at the stage of diakinesis. Such a feature has been reported by several workers (**19, 20, 25**). OLIVE (**21**), on the other hand, working on the nuclear phenomena of *Coleosporium helianthi*, noticed the persistence of the nucleoli during all the stages of the first meiotic division. It was also stated that they undergo splitting like the chromosomes and are translocated into the two daughter nuclei. Since these observations by the several investigators have been made on very carefully fixed and stained preparations, it is probable that the behavior of the nucleolus during nuclear division varies in different species of rusts.

The early stages in organization of the spindle are obscure. Its formation usually coincides with the disappearance of the nuclear membrane in late prophase. After its organization, the translocation of the chromosomes along the spindle to the respective poles can be made out (figs. 18, 19, 21, 22). Chromosome counts made during both diakinesis and the metaphase indicate that the haploid number is six. Chromosome numbers are also known for some other rusts; about eight in *C. helianthi* (**21**), eight for *Cronartium ribicola* (**8**), six to ten in *Coleosporium sonchi-arvensis* (**14**), and five in *Puccinia malvacearum* (**2**) (inaccurately described as two by many previous investigators). The presence of a centrosome with the characteristic polar radiations and other amphiastral figures has not been noticed. SAVILE also failed to find them in any of the rust species investigated by him. Centrosomes with polar radiations have been figured by COLLEY for *Cronartium ribicola* and by OLIVE for *Coleosporium helianthi*. In the latter rust OLIVE attributed to them the function of contributing toward the formation of the cell plate. The presence or absence of the centrosomes and polar radiations might be characteristic features of the particular rust species.

Toward the later part of the anaphase the chromosomes show tendencies to become clumped (figs. 20, 23) at the poles. In many cases they were massed on either side of the equatorial region of the spindle itself (fig. 22). The telophase is soon recognized, the nucleoli and the nuclear membrane reappearing. In a short time the second meiotic division is initiated, resulting in the formation of four free nuclei. The spindles in this second division are much smaller than in the first, with the chromosomes being too minute to permit definite counting.

INOCULATION EXPERIMENTS

AJREKAR and TONAPY by a series of inoculation studies proved the autoecious nature of *U. aloës*. They further stated that the promycelial cell with its threadlike outgrowth might sooner or later become separated from the empty part of the promycelium and actually function as a basidiospore. In the present studies, germinating teliospores were placed on young leaves of *A. vera*, and the inoculated plants were inclosed in moist chambers for 48 hours, after which they were placed in a greenhouse for

observation. The inoculated portions of the leaves were fixed at different intervals (24, 48, and 72 hours after removal from the moist chambers), and microtome sections were cut to observe the various stages of penetration into the host. The whiplike germ tube, upon contact with the epidermis of the host, first forms an appressorium and enters the epidermal cell by the formation of a slender process (fig. 24). It is probable that all three nuclei enter into the host along the infection hypha, but no definite observations have been made to this effect. The three nuclei have not been recognized individually because of the fact that soon after entry they divide, and a septate mycelium is developed. This is composed entirely of uninucleate cells (fig. 25). The mycelium ramifies in the intercellular spaces, and later on the hyphae are grouped together to form the pycnial initials.

The formation of sporidia is completely suppressed irrespective of the conditions under which germinations are carried out. It is, therefore, difficult to think of monosporidial infections in the usual sense of the term. However, as each teliospore on germination has been observed to produce only one fertile promycelial cell which is equivalent with the sporidia, single teliospore infections would achieve the same end as monosporidial infections. For this purpose, single teliospores were easily picked out by adopting the dry-needle method of HANNA, and they were used for inoculating healthy leaves of *A. vera*. Successful infections were observed in ten cases, indicated by the appearance of tiny yellow specks. These gradually expanded into patches, and the development of pycnia, followed by telia, took place after 14–20 days. If these single teliospore inoculations could constitute monosporidial infection—which cannot be claimed with certainty—then it might be presumed that *U. aloës* behaves like a homothallic rust.[1] The cytological details observed during teliospore germination indicate that the nuclear cycle is not of the usual type found in *Puccinia malvacearum* but is somewhat complicated.

Discussion

In rusts and in many of the higher basidiomycetes a species is considered to be homothallic when the entire life-cycle is completed starting from a single uninucleate basidiospore. Numerous microcyclic rusts have been studied experimentally (4) and their homothallic or heterothallic nature confirmed. In *P. malvacearum* (3) the basidiospore is uninucleate and on infection develops a uninucleate mycelium. Binucleate cells appear at various points in the initiation of the telial stage, and the two nuclei which associate in a young teliospore are the products through successive mitoses of the original sporidium. According to JACKSON (15), homothallic rusts were derived from heterothallic ancestors through simplification of the life-cycle. Two kinds of homothallism are distinguished (6): those that are haplomonoecious like *P. malvacearum*, where the life-history starts with a single uninucleate sporidium, and those that are sexually heterokaryotic or miktohaplontic (16, 17). In the miktohaplontic species, the ascospore or the basidio-

[1] In a recent paper JACKSON (Trans. Roy. Soc. Canada, Sec. V, Ser. III, 38:1–32. 1944) stated that the two haploid thalli developed from + and − basidiospores are not of different sex but must be interpreted as bisexual (sexually homothallic) hermaphroditic, intrasterile, and cross-fertile. If *Puccinia graminis* is then characterized as being homothallic, according to this interpretation, DODGE (Mycologia 37:784–791. 1945) pointed out that miktohaplontic forms like *Neurospora tetrasperma* would be doubly homothallic.

spore initially is bisexual, containing the nuclei of both sexes. Among the ascomycetes *Neurospora tetrasperma* (9), *Pleurage anserina* (12), and *Gelasinospora tetrasperma* (13) are miktohaplontic. In two species of basidiomycetes—*Puccinia arenariae* (18), and *Cintractia montagnei* (23)—the promycelium remains two-celled; the two basidiospores receive two nuclei each and thus are considered bisporous. These and other basidially bisporous forms of Hymenomycetes like *Coprinus ephemerus* and *Galera tenera* (24) were grouped under miktohaplontic species by BULLER (6). Considering the life-cycle of *U. aloës*, it is evident that the basidium remains mostly two-celled, the upper cell containing three or rarely two nuclei derived after the meiotic division in the promycelium. It is probable that this is a case similar to that of *Cintractia montagnei*, *Puccinia arenariae*, and others which are considered by BULLER as basidially bisporous.

The degeneration of one or two nuclei in the lower cell of the promycelium in *U. aloës* is rather enigmatic. Usually in rusts after the meiotic divisions in the promycelium, the four nuclei are distributed in four haploid or two diploid sporidia. The degeneration of any of the nuclei would mean a loss of certain combinations of characters. Such a phenomenon, however, has been observed in some of the basidiomycetes of which two instances are cited.

a) *Endophyllum valerinae-tuberosae* is a homothallic rust with a uninucleate mycelium. In the young teliospore two nuclei are present, but no synkaryon is formed, one of the two nuclei degenerating. The remaining nucleus migrates into the promycelium, and a mitotic division results in a two-celled promycelium. The upper cell forms a uninucleate sporidium, while the nucleus in the lower cell degenerates. Degeneration of nuclei thus occurs twice within the course of the life-cycle.

b) In *Psalliota campestris* the basidium is bisporous, each spore possessing two nuclei. BUHR (5) observed that in some basidia only a single nucleus enters each basidiospore, the other two nuclei degenerating within the basidium-body. The degeneration of one or two nuclei in the lower cell of the promycelium of *U. aloës* is comparable with the condition present in the two above-mentioned cases and might indicate an aberrant mode of the sexual process.

Since the discovery of the functions of pycnia by CRAIGIE, rust species with well-developed pycnia are usually considered heterothallic (4), and in the homothallic species the pycnia are either absent or underdeveloped. In *U. aloës* the pycnial development is incomplete in the sense that no ostiole with flexuous hyphae are produced. The pycniospores are extruded out of the narrow pore through the epidermis and collect in a droplet of secreted nectar. In some of the pycnia the entire cavity is filled by pycniosporophores without any spore development. According to JACKSON (15), "the presence of spermagonia or spermatia, however, should not be considered as indisputable evidence that the species is heterothallic. It is quite possible that in species spermatia are formed but are no longer functional." JACKSON cited as evidence the instances of *Endophyllum euphorbiae-sylvaticae* and *Gymnoconia nitens*—the form with a four-celled promycelium studied by DODGE and GAISER (10)—in which the nuclei do not fuse prior to the formation of the basidium, thus suggesting a homothallic development, and yet they show development of pycnia. *E. cen-*

tranthi-rubri and one of the uninucleate strains of *G. nitens*, which possess the haplophase throughout their life-cycles, could only be homothallic. However, pycnial development has been observed, and these must be nonfunctional. In *Cystopsora oleae* the mycelia produced as a result of sporidial or aeciospore inoculations of young leaves are first multinucleate and later on dikaryotically diploid, and yet well-developed pycnia with ostiole and paraphyses as in heterothallic rusts are present (**28**).

JACKSON (**15**), in comparing the life-cycle of *Herpobasidium filicinum* with the microcyclic rusts, enumerated six distinct types of variations present in the endo- and micro-forms. The life-cycles of long-cycled rusts are mostly uniform in all essential features. The life-cycle of *U. aloës* shows that it cannot be equated with any of the types enumerated by JACKSON and thus adds another variation.

Summary

1. *Uromyces aloës* (Cke.) Magn. is a microcyclic rust developing pycnia and telia on the leaves of *Aloe vera*. The pycnia are subepidermal and remain abortive and underdeveloped in many cases.

2. The telia are subepidermal, and the teliospores germinate readily, producing usually two-celled, and rarely three-celled, promycelia. The terminal cell, which may thus be bi- or trinucleate, is fertile, while the nuclei in the lower cell, or cells, degenerate. The terminal fertile cell produces an infection hypha which penetrates into the host. Development of basidiospores has not been observed, but the terminal cell of the promycelium assumes the function of basidiospores.

3. A detailed cytological study of teliospore germination has been made. The haploid chromosome number, as determined during the meiotic divisions of the fusion nucleus in the promycelium, is six. In its type of teliospore germination, *U. aloës* has been shown to resemble *Puccinia arenariae, Cintractia montagnei*, and others which are considered to be basidially bisporous by BULLER.

UNIVERSITY OF WISCONSIN
MADISON, WISCONSIN

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CHARACTERISTICS OF THE NITROGEN-FIXING ENZYME SYSTEM IN NOSTOC MUSCORUM[1]

R. H. BURRIS AND P. W. WILSON

Introduction

Fixation of molecular nitrogen by the blue-green alga *Nostoc muscorum* is of special interest, since in some respects this system represents an intermediate between fixation by the free-living heterotrophic bacterium, *Azotobacter*, and the symbiotic combination of *Rhizobium* plus leguminous plant. Previous studies from this and other laboratories (**2**, **4**, **9**), dealing with the properties of the enzyme systems responsible for nitrogen fixation by *Azotobacter* and by inoculated red clover plants (*Trifolium pratense* with *R. trifolii*), established that these two systems resemble one another in many critical respects—dissociation constant (K_{N_2}), response to H_2 and to CO. Extension of such investigations to *Nostoc* was recognized but delayed because of lack of suitable technique. Fixation by this organism is slow, and replicates often vary considerably. As studies on the nitrogen-fixing system are as yet limited to growing cultures, these defects in experimental method discouraged enzyme studies; it appeared unlikely that sufficient precision would be obtained for investigations of this nature. We have overcome to a considerable extent this handicap by use of many-fold replication and by application of the isotopic tracer method. Although the data still lack the precision achieved with either *Azotobacter* or in-

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oculated red clover plants, they are sufficiently precise for comparison with these two.

Methods

The pure culture of *N. muscorum* employed was kindly furnished by Dr. F. E. ALLISON. Inoculum for the experiments was prepared: 25-ml. portions of approximately 2-week-old cultures in Roux bottles were transferred to 100 ml. of fresh ALLISON's nitrogen-free medium with 1% sucrose (1) in 1-liter Roux bottles, and the cultures were incubated about 1 week in a south window at a room temperature of approximately 25° C. Six of these 125-ml. cultures were then combined with 800 ml. of fresh medium and shaken vigorously with glass beads in a 2-liter flask. Sterile air was passed through the flask rapidly to keep the algal clumps suspended, while 35-ml. portions were pipetted into 1-liter flat-bottomed pyrex bottles. Combined-nitrogen compounds were added to individual bottles as sterile neutral solutions in 1 ml. volume. The bottles were stoppered in pairs, and gases were removed and added through cotton plugs to the two bottles simultaneously. Unless otherwise noted, the pO_2 was initially 0.2 atm., and the pCO_2, 0.05 atm., in all bottles. The other gases were varied as indicated by particular experiments. The culture bottles were incubated in a greenhouse at a normal temperature of 22°–25° C.; during the late spring and summer months the temperatures exceeded 25°, and it was necessary to shade the bottles from direct sunlight. The bottles were illuminated at night by 200-watt bulbs in reflectors at a distance of 30 inches. Cultures were usually analyzed after incubation for 4–7 days. The cells from flasks to which combined nitrogen was added were washed by centrifugation before Kjeldahl analysis.

Experimentation

THE pN_2 FUNCTION

The partial pressure of nitrogen (pN_2) was varied from 0.02 to 0.75 atm.; helium was added to atmospheric pressure, correction being made for the 2.5% N_2 in the helium sample. Each treatment was replicated four times in the nitrogen-fixation series and twice in the combined-nitrogen series (300 p.p.m. NH_4^+–N). Variations arising from lack of uniformity in inoculum, in alterations of growth rate through disturbing the algal pad, and from differences in light should be partially eliminated by averaging of the replicates. Data from four trials are summarized in table 1. Considerable variation in response still exists both within and among the experiments even when the averages are used. In general, the data agree with those obtained in similar experiments made with *Azotobacter* (10) and with red clover plants (9), but two quantitative differences are suggested.

First, there is some evidence that assimilation of fixed nitrogen is not entirely independent of the pN_2. If true, it indicates that the level of NH_4^+–N used was insufficient to inhibit completely nitrogen fixation, a result quite different from that noted with the other agents (11). Because of its implication for the mechanism of fixation, this suggestion is worth keeping in mind, though the evidence is certainly not conclusive, since the differences are neither marked nor consistent in the sense that uptake of NH_4^+–N always decreased when the pN_2 was lowered.

Second, the dissociation constant (K_{N_2}) appears to be at least 0.10 atm. (see data in last column of table 1; value of the pN_2 at which fixation is one-half maximum). With *Azotobacter* (10) the

value is 0.01–0.02 atm. and with red clover, about 0.05 atm. (9). If the data are plotted for estimation of K_{N_2} ($1/pN_2$ against $1/v$) decision must be made as to the best measure of v, the velocity constant of fixation. As insufficient data are available to decide this definitely, calculations were made of two possibilities: (*a*) a linear rate, i.e., the final total nitrogen fixed was used as a measure of v; (*b*) a logarithmic rate in which the first-order velocity constant, $k = 2.303/\text{time}$ in

TABLE 1*

ASSIMILATION OF N_2 AND NH_4^+–N AS FUNCTION OF THE pN_2 BY NOSTOC MUSCORUM

pN_2 (ATM.)	NH_4^+–N ASSIMILATED (MG./35 ML.)				N_2 FIXED (MG./35 ML.)				
	Experiment†			Av.‡	Experiment†				Av.‡
	1	2	3		1	2	3	4	
0.02	3.95	.....	5.98	95	0.57	0.61	0.55	.....	28
.05	3.88	.....	5.20	84	0.93	0.79	0.89	0.54	43
.10	.....	6.14	4.57	77	0.81	1.00	1.08	0.48	47
.20	3.38	7.11	6.23	90	1.22	1.28	1.04	0.74	57
.40	4.02	7.01	5.29	89	1.16	2.16	2.03	0.78	85
0.75	4.41	7.87	6.02	100	1.81	2.67	1.85	1.63	100

* Values in table corrected for nitrogen at start. Blank indicates not included or sample lost.

†

Expt.	Time	Mg. N at start
1	7 days	0.51
2	4 days	.44
3	5 days	.45
4	4 days	0.25

‡ Relative to value at 0.75 atm. = 100.

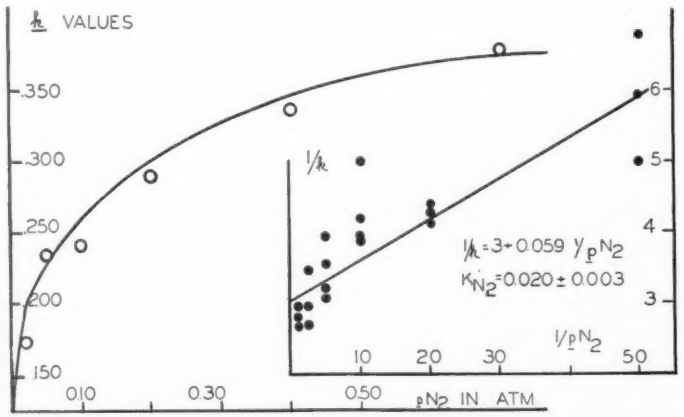

FIG. 1.—Response of *Nostoc muscorum* to partial pressure of nitrogen in atmosphere. Values of k in each experiment adjusted to a common k_{max} for the combined plotting. Each point is average of three to four separate determinations, each of which is based on four replicates.

Insert: Calculation of K_{N_2} from these data. Individual determinations shown.

days×log (final N/ initial N), was used. The following values for the K_{N_2} were obtained under the first assumption: 0.028, 0.042, 0.041, and 0.058 atm. Combining the data from all experiments led to a value of 0.032 atm., but the appropriate statistical test (**6**) indicated that a linear function did not properly describe the combined data, so this value is suspect.

The data based on an assumed logarithmic rate are shown in figure 1. In this case the combined data showed less evidence of nonlinearity, but a more desirable fit to a straight line is still needed; the F value (**6**) was just at the 5% level of significance. Also, several of the observed rates at the higher partial pressures of N_2 (0.40 and 0.75 atm.) are greater than the estimated k_{max}. Although the excesses are not more than can be accounted for by experimental error, their existence casts doubt on the accuracy of the estimated K_{N_2}. For these reasons the indicated value, 0.02 ± 0.003 atm., is highly provisional, and its close agreement with that observed with *Azotobacter* (**10**) is likely only fortuitous.

Experiments concerned with influence of CO on the fixation process included four in which partial pressures of N_2 of 0.75 and 0.10 atm. were compared in the absence of the inhibitor. The average value of k for the former was 0.414 and for the latter, 0.268. Calculation of K_{N_2} from the formula (**10**)

$$K_{N_2} = \frac{k - k'}{k'/p' - k/p}$$

for these four trials led to a value of 0.07 ± 0.019 atm. (cf. with red clover equal to 0.05 atm.). Although a more precise evaluation of K_{N_2} for the *Nostoc* system awaits additional experiments in which the rate of reaction is determined by periodic analyses, present estimates justify the conclusion that this significant enzyme constant does not differ markedly from those of the nitrogen-fixing systems in *Azotobacter* and *Trifolium*.

TABLE 2

EFFECT OF H_2 ON UPTAKE OF FREE AND COMBINED NITROGEN BY NOSTOC MUSCORUM (ISOTOPIC EXPERIMENTS)

Expt.	Time in hours	pN_2 (atm.)	pO_2 (atm.)	Atom % excess N^{15}	
				Vacuum	0.6 atm. H_2
5	70	0.16	0.24	0.267 0.224 0.313	0.172 0.179 0.182
6	144	.20	.20	5.44	5.20
7	36	.20	.20	0.171	0.050
8*	28	0.20	0.20	6.43 6.60 6.82	6.74 6.46 6.61

* 50 p.p.m. NH_4^+–N (32% atom excess N^{15}) added in this experiment.

THE pH_2 FUNCTION

A critical test for comparison of nitrogen-fixation systems is their response to molecular hydrogen. Although this gas is inert for most enzyme systems other than those involving hydrogenase, it *specifically* and *competitively* inhibits nitrogen fixation by both *Azotobacter*

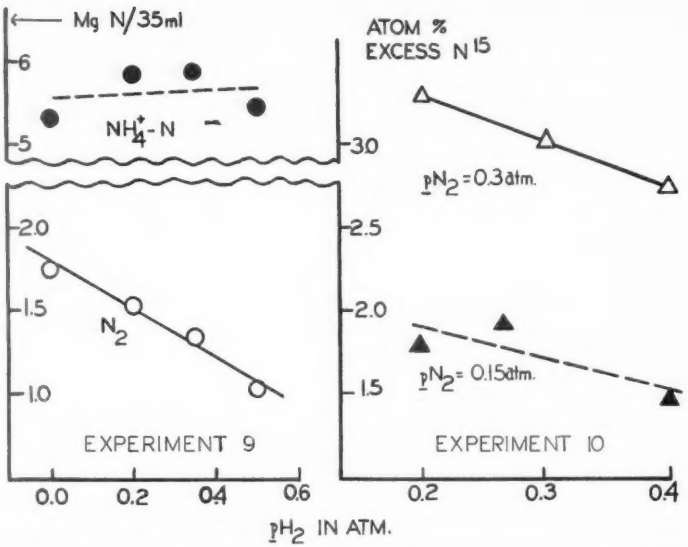

FIG. 2.—Effect of hydrogen gas on uptake of free and combined nitrogen by *Nostoc muscorum*. *Left*, comparison of assimilation of N_2 and NH_4^+-N (total-nitrogen experiment, $pN_2 = 0.2$, $pO_2 = 0.175$ atm., helium to fill). *Right*, effect of pN_2 on action of H_2 (isotopic experiment, $pO_2 = 0.3$ atm., vacuum).

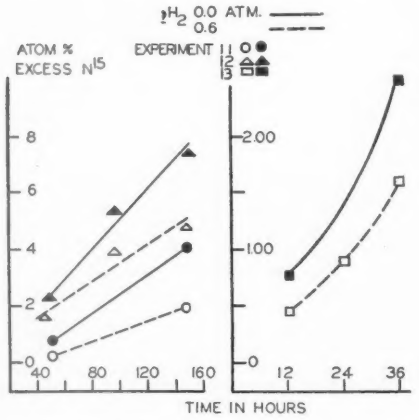

FIG. 3.—Effect of hydrogen on rate of nitrogen fixation by *Nostoc muscorum*.

(13) and red clover (9). Because of the importance of the effect of hydrogen, a more sensitive method was sought for verification of data obtained in the total-nitrogen type of experiment. It was found that many of the irregularities could be eliminated if isotopic N^{15} were used to measure fixation as has been previously described (3). The absolute quantity of cells initially added as inoculum is not especially critical in governing the amount of N^{15} they assimilate, and the experiments can be completed in a relatively short time, minimizing confusing secondary reactions. The chief limitation of the method at present results from the lack of sufficient equipment to handle numerous replicates in a single experiment. For this reason we have not used it in the pN_2 experiments, although the procedure is certainly recommended whenever facilities become available.

For test of the method *Azotobacter* cultures were grown in the presence and absence of H_2 (0.6 atm.); the excess of N^{15} found in the cultures after 28 hours over that normally present was: without

H_2, 3.52, 3.44, and 3.44; with H_2, 2.66, 3.06, and 2.67 atom %. In a second experiment after 19 hours the values obtained were: without H_2, 1.45, 1.43, and 1.66; with H_2, 0.96, 1.07, and 0.70 atom %.

Data in table 2 and in figures 2 and 3 demonstrate that H_2 likewise specifically inhibits nitrogen fixation by *N. muscorum*. This is evidenced by the results obtained with N^{15} (table 2, figs. 2, 3) as well as by those with the more traditional total-nitrogen method (fig. 2, expt. 9). The specificity of the effect is illustrated by the data of experiments 8 (table 2) and 9 (fig. 2), which indicate that the effect of H_2 is confined to assimilation of N_2 and has little, if any, effect on uptake of NH_4^+–N. Whether the effect is competitive or not cannot be stated without further additional study, but the results of experiment 10 are consistent with the view of competition. The results of experiments 11, 12, and 13 (fig. 3) suggest that, as previously noted with the other nitrogen-fixing agents, the effect is on the rate of fixation. From these results with H_2 it is concluded that the nitrogen-fixing system in *Nostoc* is closely related to that in *Azotobacter* and in red clover.

The *p*CO function

Further evidence of the similarity of the nitrogen-fixation system in *Nostoc* with that in *Azotobacter* and in red clover is furnished by experiments dealing with the effect of CO. This gas does not possess the specificity for inhibition of fixation shown by H_2, since it may affect the assimilation of nitrate and, at relatively high concentrations, of ammonium (12). Nevertheless, because of quantitative differences in the effect on fixation of N_2 as compared with assimilation of the combined forms, its use as an inhibitor provides another valuable criterion for establishing similarities and differences among biological nitrogen-fixing systems.

TABLE 3

Effect of CO on fixation of N_2 by Nostoc muscorum (isotopic experiments)

Expt.	Time in hours	*p*CO (atm.)	Atom % excess N^{15}
$p^{14}O_2$=0.2 atm. pN_2=0.2	38	0.00	0.108 .131 .138
		.006	.039 .033 .035
$p^{15}O_2$=0.2 atm. pN_2=0.2	28	.000	.115 .144
		.0005 .002 0.004	.107 .060 0.009

TABLE 4

Action of CO on the assimilation of ammonium nitrogen by Nostoc muscorum (isotopic experiment 16)

Atmosphere	Atom % N^{15} excess
Air	8.17
Air + *p*CO 0.0005*	8.26
Air + " 0.001	8.41
Air + " 0.002	8.52
Air + " 0.004	8.09
Air + " 0.006	8.42
Air + " 0.02	8.33
0.80 vac., 0.004 CO, 0.20 O_2	7.58
0.80 H_2, 0.004 CO, 0.20 O_2	7.95
0.80 He, 0.004 CO, 0.20 O_2	7.78

* All values in this column expressed as atm.

The response of *N. muscorum* to CO was determined in isotopic and total-nitrogen experiments; the results are summarized in tables 3–6 and in figure 4. The specificity of the action of CO at a *p*CO of 0.006 atm. (0.6%) or less is

demonstrated by comparison of the data in tables 3 and 4. The ammonium ion in experiment 16 (table 4) was supplied as 50 p.p.m. $(NH_4)_2SO_4$ containing 32% atom excess N^{15}. After 24 hours' incubation the cells were recovered by centrifugation, washed, and analyzed for N^{15}. Since the level of the heavy isotope was approximately the same in all samples, it is concluded that CO at the concentrations employed has no effect on assimilation of NH_4^+ and is therefore specific for fixation of N_2. Similar results were obtained in experiments 17–22 (tables 5, 6) which were based on total-nitrogen data and which included both nitrate and ammonium nitrogen. A rate experiment is shown in the insert of figure 4.

In general, the response of *Nostoc* to CO is similar to that already observed in *Azotobacter* (**7, 12**) and red clover (**8**). The effective levels are of the same order of magnitude, and the inhibition at these low pCO values appears to be restricted to fixation of N_2. The differences between the influence of CO on uptake of NO_3^- and NH_4^+ established in respirometer experiments with *Azotobacter* (**12**) are not too much in evidence (table 6), but, in view of the experimental error and the relatively long time required for the total-nitrogen experiments, the expected comparatively small quantitative difference could be readily hidden. More important differences are suggested by the data dealing with the nature of the inhibition shown in figure 4, in which the average values from four experiments with a pN$_2$ of 0.10 atm. and from

TABLE 5

EFFECT OF CO ON ASSIMILATION OF FREE NITROGEN (TOTAL-NITROGEN EXPERIMENTS)

pCO (atm.)	Experiment 17 (96 hr.)*		Experiment 18 (96 hr.)*	
	N_2 fixed/35 ml. (mg.)	Relative	N_2 fixed/35 ml. (mg.)	Relative
0.000	2.09	100	1.11	100
.00025	2.67	127	1.17	105
.0005	1.54	74	1.06	90
.00075	1.83	87	1.06	90
.0010	1.84	88	0.89	80
.0015	0.89	33	0.34	31
.002	0.19	9	0.13	12
0.003	0.05	2	0.05	5

* pN$_2$ = 0.75, pO$_2$ = 0.2 atm. in both experiments.

TABLE 6

EFFECT OF CO ON UPTAKE OF COMBINED NITROGEN BY NOSTOC MUSCORUM (TOTAL-NITROGEN EXPERIMENTS)

Expt.*	pCO (atm.)	300 p.p.m. NO_3^--N		300 p.p.m. NH_4^+-N	
		Uptake/35 ml. (mg.)	Relative	Uptake/35 ml. (mg.)	Relative
19 (120 hr.)	0.000	3.29	100	6.07	100
	0.003	2.49	76	5.05	83
20 (96 hr.)	0.000	2.32	100	4.65	100
	0.003	1.86	80	4.49	96
21 (96 hr.)	0.000	2.80	100	5.35	100
	0.002	3.24	115	4.73	88
22 (96 hr.)	0.000	2.81	100	3.71	100
	0.002	2.68	95	3.40	92

* pN$_2$ = 0.75, pO$_2$ = 0.20 atm. in all experiments.

five experiments with a pN_2 of 0.75 atm. are plotted. Each point at the lower pN_2 represents eight replicates and, at the higher, fourteen replicates. Two results are noteworthy: (*a*) the quantity of nitrogen fixed at a pCO of 0.0005 atm. and a pN_2 of 0.75 atm. was consistently greater than that in the absence of CO and (*b*) competitive inhibition is indicated, since CO exerted a greater percentage inhibition at the lower pN_2. A by *Nostoc* (Michaelis dissociation constant, K_{N_2}), although not determined with great precision, apparently does not differ markedly from those observed for *Azotobacter* (0.02 atm.) and red clover (0.05 atm.).

2. The response to H_2 and CO is much the same as with the *Azotobacter* and the symbiotic system. The data indicate considerable unity in the properties of the nitrogen-fixation systems in the three

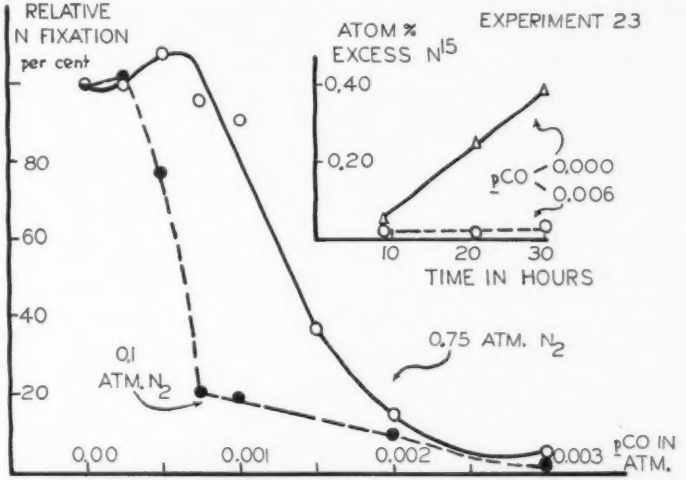

FIG. 4.—Effect of CO on fixation of nitrogen by *Nostoc muscorum* at different pN_2. (expts. 24–28). *Insert:* Time course of effect of CO measured by fixation of N^{15}.

stimulative action of CO has not been observed with either *Azotobacter* or red clover, and the inhibition of *Azotobacter*, at least, is largely, if not entirely, noncompetitive (5).

Summary

1. The properties of the nitrogen-fixing enzyme system in *Nostoc muscorum* appear to be very similar to those already noted in the free-living bacterium, *Azotobacter* and in the symbiotic system of *Trifolium pratense* and *Rhizobium trifolii*. The half-maximum rate of fixation types of organisms. The specific activity of CO in very low concentrations and of H_2 are unusual phenomena; this adds significance to their action on the enzyme systems from different sources.

3. Although the evidence strongly suggests that nitrogen fixation is mediated by similar enzyme systems in *Azotobacter*, *Trifolium*, and *Nostoc*, one point of difference may exist which might be significant. In contrast to *Azotobacter* (and possibly red clover), CO is more effective as an inhibitor of N_2 fixation by *Nostoc* at a pN_2 of 0.10 atm.

than at a pN_2 of 0.75; this is suggestive of competitive inhibition. The half-maximum rate of fixation occurs at approximately a pCO of 0.0006 (pN_2 of 0.10 atm.) and at a pCO of 0.0014 (pN_2 of 0.75 atm.). These values are intermediate between those noted with *Azotobacter* (0.004–0.005 atm.) and with red clover (0.0002 atm.).

DEPARTMENT OF AGRICULTURAL BACTERIOLOGY
UNIVERSITY OF WISCONSIN
MADISON, WISCONSIN

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CYTOLOGICAL STUDIES IN THE ALISMACEAE

WALTER V. BROWN

Introduction

The present study was undertaken to determine whether cytology can be of aid to a clearer understanding of the taxonomically difficult genus *Sagittaria* and to determine with more certainty the chromosome numbers and morphology of species in this genus and *Alisma*.

OLESON (9) reviewed the literature concerning chromosome numbers in this family through 1941. Het count for *Alisma plantago*, $2n=14$, is at variance with those of LIEHR (4), $2n=12$, and of WULFF (13), $2n=10$. HAGERUP (2) reported this species to have $n=7$ and *A. lanceolatum* $n=14$, thus confirming OLESON's determination. In *Sagittaria*, also, a range in numbers has been reported. LIEHR counted $2n=16$ for *S. sagittifolia*, TAYLOR (11) $2n=ca.$ 20 for *S. montevidensis*, NAWA (8) $2n=20$ for *S. trifolia* var. *sinensis*, LOHAMMAR (5) $2n=22$ for *S. sagittifolia* and *S. natans*, MORINAGA and FUKUSHIMA (6) $2n=22$ for *S. trifolia*

var. *sinensis*, and SHINKE (10) $2n=22$ for *S. aginashi*. OLESON found two more species to have $2n=22$—*S. latifolia* and *S. rigida*. All these workers studied sections of root tips and stated that they encountered considerable difficulty in counting the long and twisted chromosomes.

Material and methods

Mitotic chromosomes were studied in all species, smears being made of the root tip in aceto-carmine. Inasmuch as it was desired to spread the chromosomes a great deal, very little tissue was used on each slide, and considerable pressure was exerted on the cover slip to break the cell and, to a limited extent, to scatter the chromosomes. Absolute-acetic was used as the fixative (7 parts absolute alcohol to 3 parts glacial acetic acid) for a short time only, one-half hour to an hour. The material was smeared immediately after and directly from the fixative. All plants studied were collected by the author, except *S. arifolia* and *A. subcordatum*, from Colorado Springs, Colorado, furnished by Mr. ROBERT LIVINGSTON, and *S. sagittifolia*, which was purchased. Potted plants were grown in an artificial pool in the greenhouse at Duke University for ready access to material and then grown to maturity for herbarium specimens, which are in the author's personal herbarium.

Observations and discussion

ALISMA

The chromosome numbers of all genera and species of Alismaceae so far studied cytologically are listed in table 1. The author's counts of $2n=14$ (fig. 6) for *A. triviale* and $2n=28$ (fig. 4) for *A. subcordatum* substantiate the counts of OLESON and HAGERUP. It is probably true, as OLESON stated, that the basic number in this genus is seven. The author disagrees, however, with OLESON's findings on the morphology of the chromosomes of *Alisma*. She stated that "they may be classed as long, medium and short." This is true, but, as figures 1, 2, 4, and 6 show, these categories are arbitrary and practically useless. The author finds a fairly regular series of chromosomes decreasing in length from about 22 to 8 μ. OLESON further stated: "Four chromosomes are long, 2 having median and 2 subterminal constrictions; 6 of these are of medium length and 4 are short." This is quite different from the author's findings. Whereas OLESON reported two long chromosomes with median constrictions, the author finds that ten chromosomes have the constriction so located (fig. 6). OLESON's four short chromosomes are the only ones with subterminal constrictions in the author's material.

FERNALD (1) recently revised the taxonomic concept of American species of *Alisma*. He finds that American *Alisma* is distinct from the European *A. plantago-aquatica* L. There are two species of *Alisma* in the United States, *A. triviale* Pursh, the more northern species with larger flowering parts, and *A. subcordatum* Raf., more southern with smaller flowering parts. OLESON's *A. plantago* is probably *A. triviale*, the diploid species, as found in the present investigation. *A. subcordatum* is tetraploid. HAGERUP reported the European species *A. lanceolatum* to be tetraploid with $n=14$. Thus, there is one diploid and one tetraploid species in Europe and one diploid and one tetrapoloid species in North America. The polyploid *A. subcordatum* appears to have two sets of chromosomes, each of which is very simi-

lar in shape and length to the set found in *A. triviale* (cf. figs. 4 and 6 as well as figs. 1 and 2).

SAGITTARIA

In *Sagittaria*, as in *Alisma*, there has been disagreement as to both chromosome number and morphology. The reports of SHINKE, LOHAMMAR, MORINAGA and FUKUSHIMA, and OLESON agree that the various species studied by them have $2n = 22$. The counts of LIEHR and NAWA have since been checked and corrected by later investigators. The count of Taylor, $2n = ca.$ 20, is indefinite, as the author himself states. The present paper reports the numbers in fifteen species of *Sagittaria* (table 1); all are the same—$2n = 22$. Nineteen species have now been studied cytologically with no polyploidy or variation in chromosome number between species.

TABLE 1

CHROMOSOME NUMBERS IN ALISMACEAE

Genus and species	n	$2n$	Authority
Alisma plantago L.		12	LIEHR (4)
A. plantago L.		10	WULFF (13)
A. plantago L.		14	OLESON (9)
A. plantago-aquatica L.	7		HAGERUP (2)
A. triviale Pursh		14	BROWN
A. lanceolatum With. = A. stenophyllum Asch. & Gr.	14		HAGERUP (2)
A. subcordatum Raf.		28	BROWN
Echinodorus ranunculoides L.	9		HAGERUP (2)
Limnophyton obtusifolium (L.) Miq.	12		NARASIMHA MURTHY (7)
Sagittaria montevidensis Cham. + Schl.	*ca.* 20		TAYLOR (11)
S. stagnorum Small		22	BROWN
S. kurziana Gluck		22	BROWN
S. kurziana X stagnorum		22	BROWN
S. subulata var. gracillima J. G. Smith		22	BROWN
S. natans Pallas		22	LOHAMMAR (5)
S. teres Wats.		22	BROWN
S. graminea Michx.		22	BROWN
S. falcata Pursh		22	BROWN
S. lancifolia L.		22	BROWN
S. rigida Pursh		22	OLESON (9)
S. rigida Pursh		22	BROWN
S. weatherbiana		22	BROWN
S. arifolia Nutt.		22	BROWN
S. latifolia Willd.	11	22	OLESON (9)
S. latifolia Willd.		22	BROWN
S. pubescens Muhl.		22	BROWN
S. longirostra (Micheli) J. G. Smith		22	BROWN
S. engelmanniana J. G. Smith		22	BROWN
S. sagittifolia L.		16	LIEHR (4)
S. sagittifolia L.		22	LOHAMMAR (5)
S. sagittifolia L.		22	BROWN
S. trifolia var. sinensis (Sims) Makino		20	NAWA (8)
S. trifolia var. sinensis (Sims) Makino		22	MORINAGA and FUKUSHIMA (6)
S. aginashi Makino	11	22	SHINKE (10)

All species appear to have the same type of genom. TAYLOR, MORINAGA and FUKUSHIMA, and SHINKE agree that there is one pair of long chromosomes with median constriction. OLESON found two pairs with median constrictions, as did LOHAMMAR. SHINKE's description of the chromosome complement of *S.*

FIGS. 1–9.—Figs. 1–3, idiograms of: fig. 1, *Alisma triviale;* fig. 2, *A. subcordatum;* fig. 3, *Sagittaria.* Figs. 4–9, chromosome complements of: fig. 4, *A. subcordatum;* fig. 5, *S. lorata;* fig. 6, *A. triviale;* fig. 7, *S. graminea;* fig. 8, *S. latifolia;* fig. 9, *S. falcata.* All chromosome drawings reproduced at ×800.

aginashi—two very long chromosomes having central fiber-attachment constrictions, eighteen rod shaped with subterminal constrictions, and two very short ones rod shaped but fiber-attachment point not designated—is the most nearly correct description in the literature. Add that the very small chromosome has a submedian constriction, and he has described the chromosome set as found in all species of *Sagittaria* yet studied cytologically. Figure 3 represents a typical or average idiogram for *Sagittaria* based on measurements of the complements of ten species and on one or more sets for each species. The study was not set up to determine and measure chromosome length with great accuracy, nor were sufficient chromosome sets of each species measured. There may be significant differences in individual chromosome lengths between species, but this study did not determine that. This average idiogram consists of one very long chromosome about 22 μ long with a central primary constriction, one very short chromosome about 6.5 μ long with submedian constriction, and nine chromosomes with subterminal constrictions and varying in length: 16, 14.5, 13, 12.5, 11.5, 11, 10.5, 10, and 9 μ, respectively. The short chromosome in the complement has a submedian constriction dividing it into two arms, one of which is twice as long as the other. The shorter arm is approximately twice as long as the short arm in the chromosomes with subterminal constrictions. This chromosome is definitely not in the latter group, however, as all chromosomes with subterminal constrictions have short arms that appear almost spherical, with a diameter slightly less than the diameter of the long arm. This is not true of the short arm of this shortest chromosome.

The idiograms of *Alisma* and *Sagittaria* are quite different (cf. figs. 1 and 3). On the basis of LEWITSKY's (3) hypothesis—that primitive members of a group have mostly isobrachial chromosomes, while derived members have mostly heterobrachial—it would appear that *Alisma* is a rather primitive genus while *Sagittaria* is more advanced or derived. If four of the five isobrachial chromosomes of *Alisma* divided to form eight heterobrachial units, a complement similar in number to that found in *Sagittaria* could be formed. Similarly, a genus phylogenetically intermediate between these two would be expected to have an intermediate number of chromosomes and from two to four isobrachial members. HAGERUP (2) reported *Echinodorus ranunculoides* to have $n=9$, but the morphology of these chromosomes was not determined. It might be expected, however, to have three long isobrachial chromosomes and six with subterminal constrictions.

Summary

1. The chromosome numbers of two species of *Alisma* and fifteen species of *Sagittaria* are reported.

2. The basic number in *Alisma* is $n=7$, consisting of five isobrachial and two heterobrachial chromosomes. *A. triviale* is diploid, *A. subcordatum* is tetraploid.

3. All nineteen species of *Sagittaria* that have been studied cytologically have $2n=22$, consisting of one pair of long chromosomes with median constriction, one pair of very short chromosomes with submedian constriction, and nine pairs of chromosomes with subterminal constrictions.

4. It is probable that *Alisma* is a primitive genus, whereas *Sagittaria* is a more advanced or derived type.

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BIOLOGICAL LABORATORY
HARVARD UNIVERSITY
CAMBRIDGE, MASSACHUSETTS

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THE ROLE OF ORGANIC MATTER, ESPECIALLY MANURE, IN THE NUTRITION OF RICE[1]

JAMES BONNER

Introduction

During the course of an investigation of the effects of manure on the growth of plants, it was found that rice responded to the addition of this material by striking increases in growth. The effect of manure was first noted in sand cultures supplied with a standard inorganic nutrient and could not be duplicated by alteration of the composition of the nutrient solution within wide limits. Neither was the effect of the manure on growth of rice owing to any possible effects on structure or tilth of the substrate, since rice is grown with the roots completely submerged, so that the effects of organic matter on soil structure and hence on the aeration of the soil, which are so important with other plants, are of little significance here. The data to be discussed below show, on the contrary, that rice cultures, under the conditions obtaining in these experiments, are able to utilize organic nitrogen and that manure influences the growth of rice through its content of organic nitrogenous compounds.

[1] Report of work supported by the John Ellis Fund. The author is indebted to Mr. ELLIS and to Dr. A. D. SHAMEL for their continued interest and many helpful suggestions during the course of this work.

Material and methods

The rice used was of the variety Colusa. It was germinated in a warm greenhouse in washed river sand contained in flats, and the seedlings were used when the plants had attained a height of 15–20 cm. to the top of the tallest leaf. No nutrient was added to the seedling cultures. Experiments were set up either in 1-gallon crocks, $1\frac{1}{2}$-quart cans, or in 20-cc. shell vials, the size of container depending on the purpose of the experiment and the length of time which the plants were to be grown. With the plants in crocks or cans, sand was used as a substrate. The containers were without bottom drainage and were filled with nutrient solution so that the roots were completely submerged. The nutrient was drained out and replaced by fresh at intervals of 1–14 days in the various experiments. Plants could be grown to maturity in the 1-gallon crocks and could be grown for 30–60 days in the $1\frac{1}{2}$-quart cans. Many of the experiments were, however, done with seedling plants grown over a period of only 2 weeks, for reasons which will become apparent below. In these experiments the rice seedlings were transplanted singly to 20-cc. shell vials containing 15 cc. of nutrient solution and stoppered with corks. Each plant was held in place in a slit in the side of a cork, and each cork was further provided with a small central hole for the addition of water. These vials were contained in lots of twenty in boxes so constructed that the vials and hence the roots of the seedling were kept in darkness within the box. Plants so cultured were grown in the air-conditioned greenhouse for 2 weeks at a constant temperature of 80° F. Growth under these conditions was in general excellent, the fresh weight of plants approximately doubling during the 2-week period.

TABLE 1

REPRODUCIBILITY OF RICE PLANTS GROWN INDIVIDUALLY IN 20-CC. SHELL VIALS AND HARVESTED IN LOTS OF TEN. EXPERIMENT M-59. INITIAL WEIGHT OF PLANTS, 1.73 GM.

Row no.	Fresh weight (gm./10 pl.)	Row no.	Fresh weight (gm./10 pl.)
1	4.61	10	3.83
2	4.30	11	4.09
3	3.74	12	3.62
4	3.95	13	4.10
5	4.26	14	3.98
6	4.27	15	4.59
7	3.71	16	4.20
8	3.50	17	4.62
9	4.74	18	4.15
Av.	4.12	Av.	4.13

The degree of variability between lots of rice seedlings grown in vials is shown in table 1. Each figure represents the fresh weight at harvest of ten rice seedlings grown for 2 weeks as outlined above. The weights differ from the overall mean by an average of 7% of the mean.

The manure used in this investigation was a well-rotted, air-dry, ground, commercial steer product. The nutrient solutions used were based on those described by HOAGLAND and ARNON (**9**) and by VICKERY *et al* (**18**). The HOAGLAND solution supplies all the nitrogen as nitrate, while the VICKERY solution supplies all the nitrogen as ammonium (table 2). In both cases the nutrient was diluted to one-half of the standard concentration, since other work, not here described, has established that young rice plants have only a relatively low tolerance to salts. The initial pH of the nitrate nutrient was 5.5, of the ammonium nutrient 6.2.

Results

PRELIMINARY INVESTIGATIONS.—It is generally known that ammonium salts

are more readily utilized than nitrates by young rice plants as a source of nitrogen. This has been noted by KELLNER (11), NAGAOKA (13), KELLEY (10), ESPINO (7), TRELEASE and PAULINO (17), THELIN and BEAUMONT (16), and PARDO (14), among others. KELLEY has shown (10) that in rice-paddy soils nitrate formation is slow and that nitrogen tends to accumulate as ammonia. Preliminary experiments confirmed the preferential utilization of ammonium nitrogen by rice. Plants supplied with nitrate in the standard nutrient grew poorly and showed typical symptoms of nitrogen deficiency while those supplied with ammonium nitrogen grew vigorously and showed only slight symptoms of nitrogen deficiency. Incorporation of solid manure in the sand of sand cultures resulted in increased growth of plants either in nitrate- or in ammonium-containing nutrient solution (table 3). The manure was mixed in the sand with which the containers were filled, and the nutrient was drained out and renewed once per week. Ten per cent by volume of manure mixed in the sand gave approximately the optimum effect in increasing growth, and this effect was reflected in height, number of tillers, and weight of plants produced. Similar results were obtained in two further experiments with nitrate nutrient. In another series of experiments, the addition of manure to ammonium nutrient cultures resulted in better growth than could be achieved by variation within wide limits of the major elements in composition of the nutrient solution. For this purpose in one experiment use was made of a triangular design in which ammonium, potassium, and calcium were varied with respect to one an-

TABLE 2

COMPOSITIONS OF NITRATE- AND AMMONIUM-CONTAINING NUTRIENT SOLUTIONS USED

SALT*	MOLARITY IN NUTRIENT	
	Nitrate-containing nutrient	Ammonium-containing nutrient
Calcium nitrate	0.0025	.....
Calcium chloride	.....	0.0045
Magnesium sulfate	.001	.001
Potassium nitrate	.0025	.....
Ammonium sulfate	.....	.0045
Potassium dihydrogen phosphate	0.0005	0.003

* In addition, micronutrients were added to supply the following concentrations: Fe, 1 p.p.m.; B, 0.5 p.p.m.; Mn, 0.5 p.p.m.; Zn, 0.1 p.p.m.; Cu, 0.2 p.p.m.; Mo, 0.1 p.p.m.

TABLE 3

EFFECT OF MANURE ON GROWTH OF RICE IN SAND CULTURE. ONE-HALF STRENGTH HOAGLAND'S SOLUTION AS NUTRIENT. EIGHTEEN PLANTS PER TREATMENT. RANDOMIZED PLOT DESIGN. EXPERIMENT M-1. INITIAL HEIGHT OF PLANTS APPROXIMATELY 10 CM.

	HEIGHT OF PLANT IN CM. AFTER			NO. OF TILLERS AFTER 39 DAYS	DRY WEIGHT (GM./PL.) AFTER 39 DAYS
	11 days	25 days	39 days		
Control: nutrient only	10.4	13.5	17.0	1.3	2.49
5% manure in sand by volume	15.9	19.6	21.9	3.7	7.32
10% manure in sand by volume	18.4	22.6	26.8	5.9	16.92
25% manure in sand by volume	14.3	20.9	27.7	4.4	9.53

other while the anions sulphate, chloride, and phosphate were maintained at constant concentrations. Table 4 gives the compositions of the three stock solutions employed; these three solutions were combined by ninths to obtain nineteen different nutrient solutions. Rice seedlings were transplanted from flats to 1-gallon crocks, two plants per crock, and arranged in randomized design with four

TABLE 4

COMPOSITION OF THREE STOCK SOLUTIONS USED IN MAKING UP A NUTRIENT TRIANGLE FOR VARIATION OF AMMONIUM, CALCIUM, AND POTASSIUM IONS

Salt	Stock solution A* (ammonium stock millimols/liter)	Stock solution B* (potassium stock millimols/liter)	Stock solution C* (calcium stock millimols/liter)
$(NH_4)_2SO_4$	3		
$NH_4H_2PO_4$	2		
NH_4Cl	6		
$MgSO_4$	1		
K_2SO_4		3	
KH_2PO_4		2	
KCl		6	
$MgSO_4$		1	
$CaSO_4$			3.0
$Ca(H_2PO_4)_2$			1.0
$CaCl_2$			1.5
$MgSO_4$			1.0

* Each stock solution additionally received minor elements in the concentrations given in table 1.

plots. Each of the nineteen nutrient solutions was tested on eight crocks (sixteen plants). Growth took place in the greenhouse, and the nutrient was renewed every 1–2 weeks. In addition to the several nutrient solutions, a twentieth treatment was included in which manure was incorporated in the sand (10% by volume) and to which solution no. 9 (equal volumes of the three stock solutions) was added. Growth was allowed to continue until the grains were filled and mature. Because the experiment was started in late summer, flowering and seed setting took place while the plants were still relatively small. The plants were harvested, dried, and weighed. Results are given in figure 1. It is apparent that the high nitrogen treatments allowed greater growth of plants than treatments which supplied less nitrogen. However, the addition of manure allowed more vigorous growth than any other treatment in both total dry weight and yield of grain. This effect of added manure was manifest within 1 week of the inception of the experiment.

The experiments of table 3 and figure 1, confirmed in detail in four further experiments with rice in sand culture, establish an influence of manure on the growth of rice. The nature of this influence is the subject of the following sections.

GROWTH OF RICE SEEDLINGS.—In order to study the nature of the factors in manure which promote the growth of rice, use was made of rice seedlings grown in 20-cc. shell vials as described above. The influence of pH of nutrient was investigated in an experiment in which ammonium nutrient was buffered at various pH levels with phosphate buffer. Acidity of the nutrient appeared to be without great effect over the range of pH 4.5–7.0 (table 5). Similarly, growth of rice under these conditions did not appear to be limited by the concentration of available iron, since increasing the iron concentration by four times increased growth by less than 10%. Similar investigation of each of the micronutrients showed that no increase in concentration of any of them significantly affected growth under the conditions of the experiments.

Table 6 gives the results of an experiment in which additional ammonium was added (as ammonium sulfate) to the nutrient solution. Only small increases

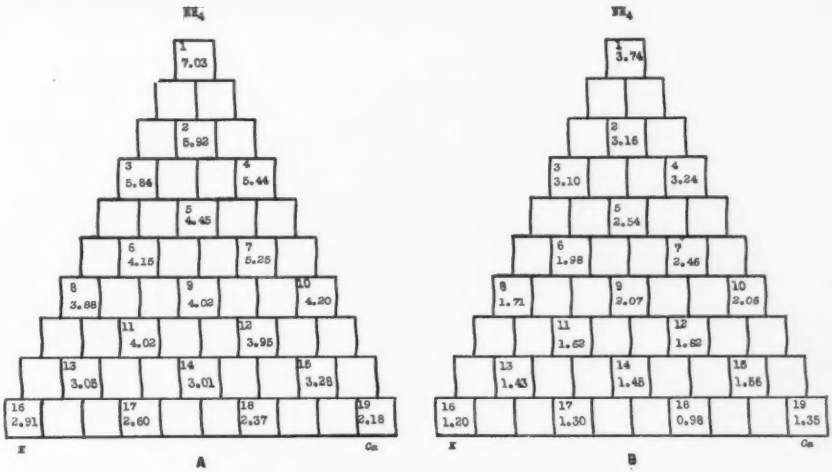

FIG. 1.—Triangular experiment on effect of nutrition on growth and seed production of rice plants in sand culture: *A*, dry weight in gm./2 pl. *B*, seeds in gm./2 pl. Solution no. 9 plus manure: dry wt., 16.86 gm./2 pl.; seed wt., 8.98 gm./2 pl.

in growth resulted from increasing the concentration of ammonium in the medium. An increase of ammonium by more than about 25% above the concentration in the basal medium resulted in the appearance of toxicity symptoms. Nitrate-nitrogen appeared to be incapable of supporting the growth of rice seedlings under the present conditions (table 11).

ACTIVITY AND FRACTIONATION OF MANURE.—Addition of manure to rice grown in vials with ammonium nutrient resulted in increased growth (table 7). Addition of 200 mg. dry weight of screened manure per rice plant increased growth, as measured by fresh weight, by 38% over the control plants after a period of 2 weeks. The manure was therefore subjected to fractionation by various procedures, one of which was exhaustive extraction with acetone in a Soxhlet extractor. Table 7 shows that the solid manure residue left after extraction is even more effective than the original manure, since an aliquot of the residue which represented 200 mg. of initial manure caused an increase in fresh weight above the controls of 106%. The material extracted by acetone, on the other hand, was found to be toxic. It would appear, therefore, that acetone

TABLE 5

EFFECT OF VARYING PH ON GROWTH OF RICE SEEDLINGS IN VIALS. 0.025 M PHOSPHATE BUFFER IN AMMONIUM NUTRIENT. EXPERIMENT M-16

PH OF NUTRIENT	NO. OF PLANTS	FRESH WEIGHT IN GM./10 PL.		
		Stems	Roots	Total
4.5	20	1.14	1.45	2.59
5.0	20	1.29	1.51	2.80
5.5	20	1.08	1.15	2.23
6.0	20	1.09	1.22	2.31
6.5	20	1.25	1.55	2.80
7.0	20	1.10	1.07	2.17
7.5	20	0.99	0.95	1.94
8.0	20	0.94	0.80	1.74

removes some toxic material from the manure, resulting in an enhancement of the growth-promoting activity of the residual acetone-insoluble material.

Only a small portion of the growth-promoting activity of manure is soluble mainly associated with the solid portion of the manure.

INFLUENCE OF KNOWN SUBSTANCES ON RICE.—The fact that water extracts of manure are ineffective in promoting growth of rice indicates that the active

TABLE 6

EFFECT OF VARIOUS CONCENTRATIONS OF AMMONIUM SULFATE ON GROWTH OF RICE SEEDLINGS IN VIALS IN AMMONIUM NUTRIENT. EXPERIMENT M-37

ADDENDUM	AMOUNT OF N AS NH_4 (MG./PL.)			FRESH WEIGHT IN GM./10 PLANTS	
	In basal nutrient	Added	Total	Total	Growth
Initial (approx.)	.....	.....	.....	1.86	.....
Final controls	1.80	0.00	1.80	3.72	1.86
+ 0.2 mg. $(NH_4)_2SO_4$	1.80	0.046	1.85	3.65	1.79
+ 0.6 mg. $(NH_4)_2SO_4$	1.80	0.14	1.94	4.16	2.30
+ 2.0 mg. $(NH_4)_2SO_4$	1.80	0.46	2.26	3.60	1.74
+ 6.0 mg. $(NH_4)_2SO_4$ (M-45)	1.80	1.40	3.20	Toxic	.....
+20.0 mg. $(NH_4)_2SO_4$	1.80	4.60	6.40	Toxic	.....

TABLE 7

EFFECT OF VARIOUS FRACTIONS OF MANURE ON GROWTH OF RICE SEEDLINGS IN VIALS. EXPERIMENT M-11. AMMONIUM NUTRIENT

TREATMENT	AMOUNT OF MANURE REPRESENTED (MG.)	NO. OF PLANTS	FRESH WEIGHT IN GM./10 PLANTS			
			Tops	Roots	Total	Growth
Initial plants (approx.)	.....	.....	.....	.....	2.23	.....
Control	.....	70	2.67	1.90	4.57	2.34
Initial manure	200	10	2.60	2.85	5.45	3.22
Water-extracted manure	200	10	2.70	2.73	5.43	3.20
Acetone-extracted manure	200	10	3.46	3.58	7.04	4.81
Acetone extract	200	10	1.66	1.12	2.78	Toxic
Water extract of acetone-extracted manure	200	10	2.74	2.39	5.13	2.90

in water, and, on the contrary, manure retained its growth-promoting activity after exhaustive water extraction. Experiments with rice in sand culture rather than in vials confirmed this water insolubility of the principle which promotes the growth of rice. The growth-promoting factor would appear to be principle of manure cannot be a vitamin of the B group such as has been found to influence the growth of certain plants in some cases (**3**, **4**, **5**). Table 8 shows that thiamin, pyridoxin, nicotinic acid, pantothenic acid, and biotin were all without influence on the growth of rice. Yeast extract, on the contrary, was ex-

ceedingly effective in increasing growth. Various other known chemical compounds were tried in numerous experiments. All nonnitrogenous substances tested were found to be ineffective (table 9). Nitrogen-containing compounds, on the contrary, were effective in increasing growth of seedling rice plants, as shown in the summarized data of table 10. Three amino acids and asparagin were effective, while peptone and egg albumin were particularly active. That it is actually the nitrogen of nitrogenous organic compounds which is of importance in the response of rice plants is indicated by the experiment of table 11. In this experiment rice plants were grown in nutrient solution containing nitrate as the source of nitrogen. In the basal nutrient essentially no growth took place during the 2-week experimental period. Addition of further nitrate to the medium was without effect. Addition of ammonium sulfate, of yeast extract, or of egg albumin resulted in substantial growth. Thus, the nitrogen of organic compounds, even of egg albumin, is apparently available to rice plants under the present experimental conditions. Similar results were obtained with seedlings grown in a nitrogen-free medium.[2] Under these conditions, manure or egg albumin was able to serve as the sole source of nitrogen for the growth of rice plants (table 12).

TABLE 8

LACK OF EFFECT OF VARIOUS GROWTH SUBSTANCES ON GROWTH OF RICE SEEDLINGS IN VIALS. AMMONIUM NUTRIENT. EXPERIMENT M-22

Addendum	No. of plants	Fresh weight in gm./10 plants		
		Tops	Roots	Total
None (control)	40	2.38	2.53	4.91
B_1, 10γ/vial	20	2.36	2.76	5.12
B_6, 10γ/vial	20	2.42	2.45	4.87
Nicotinic acid, 10γ/vial	20	2.40	2.52	4.92
Pantothenic acid, 10γ/vial	20	2.20	2.74	4.94
Biotin, 0.01γ/vial	20	2.41	2.37	4.78
Yeast extract, 40 mg./vial	20	4.48	4.99	9.47

TABLE 9

INFLUENCE OF VARIOUS NITROGEN-FREE COMPOUNDS ON GROWTH OF SEEDLING RICE PLANTS IN VIALS. AMMONIUM NUTRIENT. SUMMARY OF VARIOUS EXPERIMENTS

Substance	No. of expts. in which tested	Concentrations		Final fresh weight at highest nonlethal conc. (% of control)
		Range tested (mg./vial)	Highest nonlethal conc. (mg./vial)	
Glucose	1	10	10	92
Sucrose	2	10–40	10	50
Starch	1	10	10	92
Malic acid	3	1–10	10	91
Pyruvic acid	1	10	10	102
Malonic acid	2	3–10	3	74
Fumaric acid	3	3–10	10	102
Citric acid	5	1–10	5	102
Succinic acid	5	1–10	5	99

TABLE 10

INFLUENCE OF VARIOUS NITROGEN-CONTAINING COMPOUNDS ON GROWTH OF RICE SEEDLINGS IN AMMONIUM NUTRIENT. SUMMARY OF VARIOUS EXPERIMENTS

Substance	Expt. no.	Concentration used (mg./vial)	Growth in fresh weight (% of control)
Asparagin	M-22	10	195
Aspartic acid	M-32	3	131
Glycine	M-29	10	152
Glutamic acid	M-31	10	200
Peptone	M-39	20	232
Egg albumin	M-39	20	271

[2] The nitrogen-free nutrient used in this and subsequent experiments contained $MgSO_4$, .001 M; KH_2PO_4, .003 M; $CaCl_2$, .002 M; minor elements and iron as shown in table 2.

Table 13 gives a quantitative comparison of the effectiveness of various sources of nitrogen in supporting the growth of rice in ammonium-containing nutrient. In the experiment of table 13, good growth was made by plants growing in the basal nutrient medium which supplied 1.8 mg. nitrogen per plant as ammonium sulfate. The addition of more nitrogen as ammonium sulfate exerted but little effect on growth, particularly since the amount of nitrogen which could be added was small owing to the toxicity of ammonium sulfate (table 6). Addition of nitrogenous organic matter in the quantities found to be beneficial to growth added significantly more nitrogen. Thus, addition of 20 mg. of egg albumin per plant added 3.3 mg. of nitrogen or approximately 1.5 times as much nitrogen as could be added as ammonium sulfate without toxicity.

The data obtained thus far can then be interpreted as follows. Rice plants grown in solution or sand culture require ammonium nitrogen. The amount which they are able to utilize over an experimental period of 1–2 weeks is greater than the amount which can be supplied without toxicity under the present conditions, owing to the high concentrations of ammonium sulfate. The nitrogen deficiency thus induced can apparently be met with other forms of reduced nitrogen which may be introduced into the medium. The nitrogen deficiency can also be overcome simply by frequent periodic renewal of the nutrient solution. Table 14 shows that if the solution was renewed daily or on alternate days, substantially more growth of rice seedlings took place than when the nutrient was renewed at less frequent intervals.

It is well known that pH of the medium is influenced by the form in which nitrogen is supplied, ammonium-containing media tending to become more

TABLE 11

EFFECT OF VARIOUS FORMS OF NITROGEN ON GROWTH OF RICE SEEDLINGS IN VIALS. BASAL NUTRIENT HOAGLAND'S DILUTED 2X, NITRATE AS NITROGEN SOURCE. EXPERIMENT M-38

ADDENDUM	NO. OF PLANTS	FRESH WEIGHT IN GM./10 PLANTS			
		Tops	Roots	Total	Growth
Initial plants	70	.....	.....	1.65	.....
Final control	40	0.95	0.77	1.72	0.07
+KNO_3, 10 mg./pl.	10	1.03	0.79	1.82	0.17
+$(NH_4)_2SO_4$, 2 mg./pl.	10	2.78	1.98	4.76	3.11
+yeast extract, 40 mg./pl.	10	3.01	2.06	5.07	3.42
+egg albumin, 20 mg./pl.	10	2.66	1.73	4.39	2.74

TABLE 12

MANURE AND EGG ALBUMIN AS SOLE SOURCES OF NITROGEN FOR GROWTH OF SEEDLING RICE PLANTS IN VIALS. NITROGEN-FREE NUTRIENT. EXPERIMENT M-56

ADDENDUM	NO. OF PLANTS	FRESH WEIGHT IN GM./10 PL.			
		Tops	Roots	Total	Growth
Initial plants	30	.....	.....	1.78	.....
Final control	50	0.78	0.74	1.52	.....
+100 mg. manure	10	1.44	1.63	3.07	1.29
+20 mg. egg albumin	10	1.81	1.43	3.24	1.46

acid as nitrogen is utilized and nitrate media tending to become more alkaline under the same circumstances. The influence of organic nitrogen on the growth of rice is not, however, exerted through effects on pH of the medium, as was shown by experiments in which the medium was buffered at pH 5.5 with phosphate buffer. Under these conditions the pH of the nutrient medium changed at most by 0.5 pH unit during the growth period of 2 weeks. Egg albumin and manure exerted their characteristic effects in increasing growth in the buffered medium.

TABLE 13

COMPARISON OF EFFECTIVENESS OF VARIOUS SOURCES OF NITROGEN IN PROMOTING GROWTH OF RICE SEEDLINGS IN VIALS. AMMONIUM NUTRIENT. BASAL MEDIUM CONTAINS APPROXIMATELY 1.8 MG. AMMONIUM NITROGEN PER PLANT. EXPERIMENT M-39

Addendum	Amount of addendum	Amount of N in addendum	Fresh weight in gm./10 plants	
			Total	Growth
Initial plants	.....	.....	2.14	.....
Final control plants	None	.....	3.67	1.53
Ammonium sulfate	2 mg.	0.46 mg.	4.03	1.89
Egg albumin	20 mg.	3.30 mg.	6.29	4.15
Peptone	20 mg.	3.30 mg.	5.69	3.55
Acetone-extracted manure (from M-32)	300 mg.	3.42 mg.	6.28	4.14

THE ACTIVE PRINCIPLE OF MANURE.—Nitrogen analyses were carried out for the various nitrogenous compounds contained in the well-rotted manure. Acetone-extracted manure contained over 1% nitrogen. Of this nitrogen, 93% was in the form of hot-water-insoluble compounds, proteins, etc., and only 7% in water-soluble form. In table 13 it was shown that addition of nitrogen to rice seedlings in the form of acetone-extracted manure exerted an effect quantitatively similar to that exerted by adding the same amount of nitrogen as egg albumin. These quantitative aspects were verified in an experiment, summarized in figure 2, in which the effects of varied concentrations of manure and egg albumin were compared. In both cases addition of 1 mg. of nitrogen per plant resulted in a substantial growth increase; addition of 3 mg. gave a larger increase, while addition of 10–11 mg. was toxic. Thus, the effects of manure and of egg albumin on growth of rice are quantitatively similar, on a nitrogen basis, even though fifteen times as much

TABLE 14

EFFECT OF VARYING FREQUENCIES OF NUTRIENT RENEWAL ON RATE OF GROWTH OF RICE SEEDLINGS IN VIALS. AMMONIUM NUTRIENT. EXPERIMENT M-58. EACH FIGURE BASED ON TWENTY PLANTS

Treatment	Frequency of nutrient renewal	Fresh weight in gm./10 pl.	
		Total	Growth
Initial plants	.....	2.00	.....
Grown 2 weeks	Once/14 days	4.34	2.34
Grown 2 weeks	Once/7 days	4.28	2.28
Grown 2 weeks	Once/4 days	4.45	2.45
Grown 2 weeks	Once/2 days	5.97	3.97
Grown 2 weeks	Once/day	7.66	5.66

weight of manure is required to supply a given amount of nitrogen as is required in the form of egg albumin.

It has been shown that the water extract of acetone-extracted manure is scarcely effective in promoting the growth of rice and that the activity resides in the water-insoluble residue (table 7). As shown above, also, very little of the nitrogen of manure is water soluble. This fact is then in accordance with the view that the growth-promoting activity of manure for rice resides in the nitrogenous compounds of the manure.

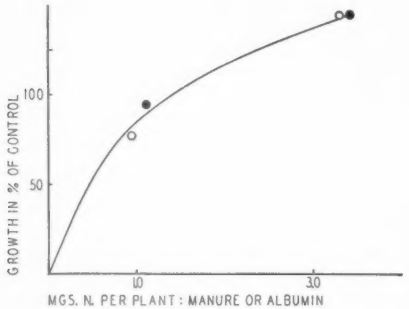

FIG. 2.—Effects of supplementary nitrogen, supplied as manure or as egg albumin, on growth of rice seedlings in vials in ammonium nutrient. Solid circles represent manure; open circles represent egg albumin.

TABLE 15

NONAVAILABILITY OF NITROGEN OF EGG ALBUMIN FOR SEEDLING RICE PLANTS GROWN IN ASEPTIC CULTURE. EXPERIMENT M-53. PLANTS GROWN IN NITROGEN-FREE BASAL NUTRIENT

Addendum	Mg./Cc.	Mgs. N per plant	No. of plants	Fresh weight after 30 days (gm./10 pl.)
None	.....	.....	40	1.23
Ammonium sulfate	0.5	1.0	40	1.68
Egg albumin	0.5	0.8	40	1.31

It would appear highly unlikely that rice plants should be able to utilize the nitrogen of protein directly. That bacteria may intervene in making protein nitrogen available for rice plants is indicated by the experiment of table 15, in which rice plants were grown under aseptic conditions in test tubes. The test tubes were approximately 1 inch in diameter by 14 inches long, and in the bottom of each 2 inches of river sand was placed. To this was added a nitrogen-free basal nutrient with or without various addenda. The whole was stoppered with cotton, autoclaved, and inoculated with aseptic rice seeds. Growth was measured as fresh weight of plants produced after 1 month. Growth in the nitrogen-free basal nutrient resulted in production of seedlings having symptoms of nitrogen deficiency. Addition of nitrogen as ammonium sulfate increased growth very considerably and relieved the symptoms of nitrogen deficiency. Egg albumin did not increase growth significantly and did not relieve the symptoms of nitrogen deficiency. Quantitatively similar results were obtained in a second experiment. It may be concluded that protein nitrogen is much less available under aseptic than under nonaseptic conditions.

Discussion

It is generally agreed that the addition of organic matter to the soil is essential to the maintenance of fertility over the years. The organic matter may be in the form of crop residues, cover crops, or farm manure; and the beneficial effects on crop yields may be determined not only in the field but in pot cultures, under experimental conditions. The nature of the effect of organic matter on soil fertility has, therefore, been the subject of intensive investigation.

Work on soils in recent years has em-

phasized the dominant role of soil structure in soil fertility. That organic matter is essential to the formation and maintenance of soil aggregates and structure has been shown by many workers and has been discussed in detail by BAVER (1), RUSSEL (15), WILLIAMS (19), MARTIN (12), and others. Whether organic matter further influences plant growth by supplying specific organic compounds which are directly utilized by the higher plant and which benefit or influence its growth in any way is debatable. It has been demonstrated beyond any doubt that many plants can grow normally in a strictly inorganic medium. It has been claimed on various occasions that addition of organic matter to inorganic nutrient media has increased the growth of one or another species of plant. In each instance that has been carefully and fully investigated, however, this claim has turned out to be only partially true, in the sense that, by alteration of the inorganic nutrient or of the environment, conditions could be found under which response of the plant to organic matter disappeared. Thus BOTTOMLEY (5) failed to provide an optimum medium for the *Lemna* with which he worked. Addition of organic matter caused increases in growth of *Lemna* under his conditions, but later workers (CLARK and ROLLER, 6) showed that even better growth could be obtained by decreasing the salt content of the medium and making provision for increased available iron. BOTTOMLEY undoubtedly supplied iron in the organic matter which he added. Recent work with *Spirodela* by GORHAM (8) has again suggested a beneficial influence of organic matter (manure), but no detailed analysis of the nature of the effect has been made. In a series of papers BONNER *et al.* (3, 4) described work which indicated that addition of certain pure organic substances increased the growth of various species of plants grown in sand culture with inorganic nutrient. These results have been confirmed only partially by the work of others. It is clear that in the original experiments special conditions of environment and nutrition prevailed, conditions under which unusually large responses to the added organic substances were obtained. In the case of *Cosmos*, which was investigated more intensively, alterations of the environmental conditions were at least as effective as the addition of organic compounds in increasing growth (BONNER, 2). Under conditions which allowed optimal growth, no effects could be obtained from added organic substances.

In the present work a systematic search was made for evidence as to whether organic matter, specifically manure, might contain special organic substances capable of causing increased growth of plants grown under the best possible environmental and nutritional conditions. Precautions were taken to rule out all effects of added manure on the physical properties of the medium. In all, sixteen species have been tested for response to manure. The tests have been carried out under a wide variety of conditions and have entailed over 120 individual experiments, each experiment in turn involving from a few to over 600 plants. Four cases were found in which the first experiment showed an apparent growth response to added manure, namely, *Poa*, *Impatiens*,[3] beet, and rice. Repetition of the experiment failed to yield a duplicate result in the case of beet, while the effect with *Impatiens* was too small for further quantitative investigation. The effect of manure on *Poa* was, however, analyzed in detail, and it was

[3] The experiments with *Poa* and *Impatiens* were carried out in co-operation with ROBERT DORLAND.

shown that *Poa*, owing to a particularly high iron requirement, suffered from iron deficiency in the conventional nutrient medium. Addition of manure merely supplied available iron, and the added growth effect of manure could be duplicated by ferric sulfate or ferric tartrate. Similarly, the effect of manure on the growth of rice has been analyzed in detail. Rice is particularly adapted to utilize ammonium rather than nitrate nitrogen, but even relatively low concentrations of ammonium are toxic to it. Several forms of organic nitrogen are available to the rice plant, including protein. Manure is shown to function in rice nutrition as a source of organic nitrogen and may serve as the sole source of nitrogen or may supplement ammonium nitrogen in the medium. Since the intervention of bacteria has been indicated to be probable in the utilization of organic nitrogen by rice, it is possible that organic forms such as protein act as reservoirs from which ammonia is slowly liberated by microbial activity.

Even in the two cases of response to manure which were found, then, the growth response on analysis has turned out to be related to the nutrition of the plant, iron nutrition in one case, nitrogen nutrition in the other. No evidence was found in the present investigation for any important role of specific organic substances contained in manure in regulating plant growth.

Summary

1. Rice plants grown in sand or solution culture were found to respond to the addition of manure with marked increases in growth. This effect of manure could not be duplicated by variations of the composition of the major or minor elements of the nutrient solution within wide limits. A systematic investigation of the nature of the effect of manure and other kinds of organic matter on the growth of rice was therefore undertaken.

2. The findings of earlier investigators that rice seedlings utilize ammonium rather than nitrate as a source of nitrogen were confirmed. In addition, it was found that ammonium nitrogen is toxic to the rice seedling at relatively low concentrations.

3. Manure and other kinds of nitrogenous organic matter are able to serve as sources of nitrogen for cultures of rice seedlings. The nitrogen of protein, peptone, and various amino acids is shown to be available to rice seedlings. These compounds are probably decomposed by micro-organisms before their nitrogen is utilized by the plant, since under aseptic conditions the nitrogen of protein does not appear to be utilized by rice.

4. The influence of manure on the growth of rice under the present experimental conditions is shown to be quantitatively attributable to the organic nitrogen content of the manure. The nitrogen of organic matter appears to serve as a supplement to the relatively low concentrations of free ammonium salts which are tolerated by the plant.

5. No evidence was found to suggest the presence in manure of a specific organic substance important in plant growth.

CALIFORNIA INSTITUTE OF TECHNOLOGY
PASADENA, CALIFORNIA

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CHANGES WHICH OCCUR IN APPLE TISSUE UPON TREATMENT WITH VARIOUS AGENTS AND THEIR RELATION TO THE NATURAL MECHANISM OF SOFTENING DURING MATURATION[1]

JEAN H. GRIFFIN AND Z. I. KERTESZ

Introduction

Changes which occur in the texture and firmness of apples on the tree and in storage are of great economic importance. In order to make apples available to the consumer during extended periods, a great proportion of the crop is placed in storage in a "tree-ripe" or relatively firm condition. The rate of softening may then be controlled, or at least retarded, by refrigeration or by modifying the atmosphere within the storage (**13**) or both.

The firmness of an apple is one of the major factors which determines its commercial value. Changes in firmness during growth and maturation (**5**) may be followed by measuring the pressure necessary to force a plunger (**10**) into the apple (after careful removal of the skin), or by determining the tensile strength, i.e., the force required to pull to pieces a sample of tissue which has been cut according to standardized procedure (**11**). There are several reports in the literature of such measurements used as an index of maturation of apples; these methods are very useful where numerical recordings are desirable.

[1] Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper no. 679.

Over one hundred years ago it was observed that unripe apples contain pectic substances which are insoluble in water and that, as ripening progresses, an increasing proportion of water-soluble pectic substances (9) appears in the tissue (1, 2). The latter are apparently formed from originally insoluble pectic constituents which are usually designated as "protopectin" and "cell-wall pectic constituents." It has been the general experience among investigators that the softening of apple tissues, whenever it occurs or however it is induced, is accompanied by pectic transformations of such a nature.

It would be wrong to assume that changes in the pectic substances are entirely responsible for the changes in texture. The size and shape of cells and undoubtedly many other factors have an influence on the firmness of the tissue. In apples the formation of new cells stops comparatively early in development and at that time the individual cells are strongly cemented together by the middle lamella which is composed of insoluble pectic materials. During maturation the middle lamella is reduced in quantity; as a result, the adhesion of the cells to each other diminishes, and thus the tissue softens. It should be also borne in mind that chewing of a tissue consists mainly of pushing the cells (or clusters of cells) apart rather than of cutting the individual cells.

Ever since the chemical changes occurring during softening of apples were first observed, it has been assumed that transformations in the pectic substances were caused by the action of enzymes. In spite of the efforts of many workers, there is no conclusive evidence that protopectinase and pectin-polygalacturonase (pectinase) (6) actually occur in apples. During the last five years many tests have been made in this laboratory by various methods to show the presence of protopectinase and pectin-polygalacturonase (pectinase) in apples, but no results proving the presence of these enzymes could be obtained.

These negative results lead to the assumption that pectic transformations in apples may occur through the action of nonenzymatic agents or chains of reactions (8). It is known that polysaccharides, including pectinic acid (pectin), may be degraded by ascorbic acid (vitamin C) or peroxides, with an especially rapid reaction taking place when both agents are permitted to act simultaneously (4). Ascorbic acid is naturally present in apple tissue (though at times in small quantities), and peroxides have also been assumed to occur in plant tissues, although likely only in a transient state. Furthermore, peroxides were shown to form during the nonenzymatic oxidation of ascorbic acid (14); thus the presence of ascorbic acid may give rise to peroxides and produce the system for degrading pectinic acids.

Knowing that ascorbic acid and peroxides, alone or in combination, are capable of degrading pectinic acids (pectin) into products which no longer come within the definition of pectic substances, it appeared that possibly the pectic changes in apples during maturation might proceed, partly at least, through this mechanism. Of even more fundamental interest is the question whether or not these chemical agents are capable of transforming insoluble pectic constituents (protopectin) into soluble ones (pectinic acids and pectins). Since no method is available for the preparation of pure protopectin, experiments were performed with the apple tissue itself. Measurements of tensile strength, followed by analyses for soluble and in-

soluble pectic constituents, were made after treatments of the tissue with ascorbic acid, hydrogen peroxide, or both. In addition, some tests were made to determine the changes in firmness and chemical composition which were caused by treatment of apple tissue with dilute HCl solutions and with a commercial pectinase[2] known to contain high polygalacturonase (pectinase) activity.

Methods

Webster apples grown on one tree in Geneva, New York, during the 1944 season were used. Apples were picked fresh for each experiment until September 20, when all fruit was picked and put into storage at 1° C. After this date they were removed, as required, from cold storage and held at room temperature overnight before using. For measurement of tensile strength a cross-section, about 10 mm. thick, first was cut from the apple. From this disk dumbbell-shaped figures were cut out by a sharp form. These dumbbells were made to exact thickness (7.8 mm.) by placing them in a Petri dish and cutting with a sharp knife sliding along the edge of the dish. The dumbbells weighed 1.9 gm. and had a cross-section at the narrowest point, where breaking occurred, of 43.7 sq. mm. For the various treatments the dumbbells were submerged, usually in pairs, in 50 cc. of solution contained in a 100-cc. beaker. Every set of samples used for tensile-strength measurements or analyses consisted of twelve dumbbells, obtained from at least three apples. The tensile-strength measurements were performed with the aid of the apparatus described by PERSONIUS and SHARP (11). Water was siphoned into the bucket weighing 146 gm. at the rate of 3.2 gm. per second until the combined weight of the bucket and water tore the center of the dumbbell apart. At this time the water was shut off and the weight of the water plus bucket measured. The twenty-four halves of dumbbells were placed in ethanol, which was boiled up and then set aside for chemical analysis.

For analysis the sample (consisting of twelve dumbbells) was ground finely with approximately 12 gm. of sand and extracted with 200 cc. of water for 3 hours at about 20° C. The sample was then centrifuged at 2500 r.p.m. for 20 minutes in a no. 2 International Centrifuge, the extract poured off, and a second similar 30-minute extraction made. These two extracts were combined, made up to 500 cc. with water, and 200 cc. of this extract was then used for the precipitation of acid-ethanol-insoluble materials by a double volume of 95% ethanol acidified to give 0.05 *N* HCl in the final mixture. After standing overnight the precipitate was filtered off, washed with ethanol, dissolved in hot water, and the pectic substances determined as calcium pectate by the POORE modification (12) of the CARRÉ-HAYNES method (3).

The residue left from the water extraction was treated with 0.05 *N* HCl for 2 hours at 85° C. The liquid was filtered off and the process repeated four times. These five extracts were combined and made up to 1000 cc. with water. The pectic substances were then determined (as above) in 200 cc. of the extract after the removal, with ptyalin, of any starch which may have been present.

Results

The results of preliminary tests (table 1) indicate that the tensile strength of apple tissue decreases throughout the season. The proportion of pectic sub-

[2] Pectinol, manufactured by the Rohm and Haas Company of Philadelphia, Pa.

stances (on the fresh-weight basis) decreased continuously, although somewhat irregularly. The percentage of water-soluble pectic substances showed an irregular tendency to increase. When the apples were held at room temperature for a week, there was a considerable drop in tensile strength and an increase in the proportion of water-soluble pectic constituents.

Treatments by various agents were performed by submerging the apple dumbbells in the solutions. It was noted that soaking in water alone decreased the tensile strength. The reason for this change must be, partly at least, the upsetting of the osmotic equilibrium in the tissue, resulting in some plasmolysis. As shown in tables 1 and 2, the effect on tensile strength of soaking in water alone was great enough to interfere with the clarity of observations on the effects of chemicals. The probable error of tensile-strength measurements in samples soaked in water was higher than in any other lot of twelve dumbbells. When buffer solutions were used, tensile strengths of the dumbbells dropped much less than in water. The important point is, however, that the decrease of tensile strength in samples soaked in water was accompanied only by a small increase in the

TABLE 1

SEASONAL CHANGES AND EFFECT OF HOLDING AT ROOM TEMPERATURE OR SOAKING IN WATER ON TENSILE STRENGTH AND PECTIC CONSTITUENTS OF APPLES (WEBSTER VARIETY, 1944)

DATE	TREATMENT	TENSILE STRENGTH (GM./MM.²)	CHANGE IN TENSILE STRENGTH (%)	PROBABLE ERROR (GM./MM.²)	PECTIC SUBSTANCES†			
					Water soluble (%)	Water soluble (% of total)	Water insoluble (%)	Total (%)
8/15		21.0	(100)	±0.6				
8/28		20.0		±0.8				0.767
9/20*		17.5	(100)	±0.7	0.066	10.6	0.555	.621
9/27	In water for 60 min.	11.5	66					
	Held 1 week	13.3	76	±0.6				
9/21		19.5	(100)	±0.5				.756
	In water for 150 min.	16.8	86					.716
	In water for 300 min.	10.4	54					
9/27		15.6	(100)	±0.7	.056	9.1	.539	.614
10/5	In water for 165 min.	10.4	67	±1.0	.090	13.9	.557	.647
	Held 1 week	5.0	32	±0.3	.089	13.8	.559	.648
10/16		13.0	(100)	±0.5	.082	11.8	.612	.694
10/23	Held 1 week	3.3	25	±0.0	.107	20.0	.448	.535
10/17		5.8			.053	9.1	.529	.582
11/30		4.6			.100	17.6	.472	.572
12/15		3.7			0.087	15.6	0.469	0.556

* Apples picked and put into storage.

† As calcium pectate, fresh basis.

proportion of soluble pectic substances, while the percentage of total pectic substances did not change materially.

Table 2 also shows some typical results obtained in tests made with ascorbic acid, hydrogen peroxide, pectinase, and HCl. In these experiments drop in tensile strength when both hydrogen peroxide and ascorbic acid were present. Although the proportion of total pectic constituents is irregular in these samples, those which were treated with both ascorbic acid and peroxide show a decrease.

TABLE 2

EFFECTS OF VARIOUS TREATMENTS ON TENSILE STRENGTH AND PECTIC SUBSTANCES OF APPLES (WEBSTER VARIETY, 1944)

Experiment no.	Date	Treatment*	Time (min.)	Tensile strength (gm./mm.²)	Probable error (gm./mm.²)	Pectic substances†			
						Water soluble (%)	Water soluble (% of total)	Water insoluble (%)	Total (%)
1	8/15	1. water	150	15.9					0.784
		2. H_2O_2 (1.5%)	"	15.1					.760
		3. AA (0.5%)	"	11.3					.855
		4. (2+3)	"	9.0					.524
		5. water	300	12.5					.751
		6. as 2	"	9.3					.718
		7. as 3	"	10.2					.807
		8. as 4	"	4.6					.467
2	9/1	Water	150	16.8		0.124	17.8	0.592	.716
		0.001 *N* HCl	"	12.6		.117	16.7	.583	.700
		0.01 *N* HCl	"	8.9					
3	9/27	(Control)		15.6	±0.7	.056	9.1	.539	.614
		1. water	165	10.4	±1.0	.090	13.9	.557	.647
		2. Buffer pH 3.3	"	12.9	±0.8	.102	13.7	.645	.747
		3. E (0.25%)	"	6.4	±0.6	.097	16.7	.483	.580
		5. AA (0.1%)	"	8.7	±0.6	.167	29.9	.492	.559
		6. AA (0.05%)	"	9.7	±0.7	.154	24.9	.465	.619
		7. H_2O_2 (3%)	"	9.4	±0.6	.259	44.0	.330	.589
		8. H_2O_2 (1.5%)	"	10.6	±0.7	.229	37.7	.379	.608
		9. (5+8)	"	7.2	±0.4	.228	50.6	.223	.451
		10 (6+7)	"	7.7	±0.4	.209	38.8	.330	.539
		11. (5+7)	"	6.8	±0.4	.170	32.6	.351	.521
		12. (6+8)	"	7.5	±0.4	0.160	31.1	0.355	0.515

* AA=ascorbic acid; E=commercial pectic enzyme (pectinase). † As calcium pectate, fresh basis.

the treated dumbbells were compared with those soaked in water or, in the last experiment (no. 3), in water and buffer solution (pH 3.3) for corresponding periods.

The first experiment showed a definite

In order to ascertain just how much the acidity of the ascorbic acid may have affected the tensile strength and the total proportion of pectic constituents, some dumbbells were soaked in solutions of hydrochloric acid. As shown in experi-

ment 2 (table 2), concentrations corresponding in pH (even if not in ionic strength) to the ascorbic acid solutions used caused some change in tensile strength but no change in distribution and total quantity of pectic constituents.

The most important set of data is that obtained in experiment 3. A 0.1 *M* phosphate buffer solution was used in all these mixtures to maintain the pH at 3.3. The use of this buffer resulted in 17% less drop in tensile strength than when water was used for the soaking (33% drop). All other solutions were made in such buffer, and all mixtures were adjusted to pH 3.3.

The enzyme treatment lowered the total pectic content, with an accompanying increase in soluble pectic constituents. Every treatment with ascorbic acid, hydrogen peroxide, or both, increased the proportion of soluble pectic constituents and decreased the percentage of total pectic constituents. These changes were in every instance accompanied by a decrease in tensile strength of the apple tissue. The drop in tensile strength and the drop in total pectic constituents are greatest where both ascorbic acid and hydrogen peroxide were used simultaneously and approximate the results obtained when the commercial pectic enzyme was used.

Discussion

The results of several investigators (4, 8) proved that ascorbic acid or peroxides, and especially ascorbic acid and peroxides together, can rapidly degrade soluble pectic compounds like pectinic acids (pectins). The relationship between protopectin, the major insoluble pectic constituent of plants, and soluble pectic substances is still not clear, but there is a distinct possibility that protopectin is rendered soluble by the action of heat, acids, or enzymes through a partial hydrolysis which would diminish its originally large molecular weight (size) (7). Treatment of insoluble pectic constituents of apples with hydrogen peroxide and ascorbic acid results in an increase in soluble pectic substances in the apple tissue. The soluble pectic constituents are then further degraded into compounds which will no longer come under the definition of pectic substances. It seems that in our experiments the latter reaction was slower than the rate of solubilization of the insoluble protopectin, since both the absolute amount and the proportion of soluble pectic constituents increased. Unfortunately, the mechanism of oxidative degradation by ascorbic acid and peroxides is yet so unclear that further speculation on these reactions is hardly warranted.

Obviously, the treatments used in these experiments resemble only to a limited degree those processes which may occur naturally in the plant. Concentrations of ascorbic acid and peroxide used in the experiments could not be assumed to be present in the fruit tissue. However, the purpose of the experiments was not an imitation of the changes *as* they occur in nature but rather to determine whether the changes which take place in the pectic constituents of apples during growth and maturation *could be* artificially brought about by the nonenzymatic agents used. According to the results obtained, this has been accomplished. As apples soften, the total pectic constituents decrease and the proportion of soluble pectic materials increases. Similar changes have been brought about in the tissue by the use of ascorbic acid, peroxide, or both. In nature these transformations occur during periods of weeks or months rather than

hours and thus might require much lower concentrations of the chemical agents than those used in the tests.

Summary

1. During the maturation and softening of apples the tensile strength of the tissue and the proportion of the total pectic constituents decrease and the proportion of water-soluble pectic materials increases. The same changes may be brought about by treatment of apple tissue with ascorbic acid or hydrogen peroxide and at a much faster rate by a combination of both agents.

2. The results indicate that, in addition to being able to decompose water-soluble pectinic acids (pectins), these agents are also able to solubilize the insoluble pectic constituents (protopectin) of apples.

3. While it appears that both protopectinase and polygalacturonase (pectinase) are absent from apples, the functions attributed to both enzymes may be performed by the nonenzymatic agents used in these experiments.

NEW YORK STATE AGRICULTURAL
EXPERIMENT STATION
CORNELL UNIVERSITY
GENEVA, NEW YORK

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PRELIMINARY RESULTS WITH THE USE OF 2,4-DICHLOROPHENOXYACETIC ACID AS A SPRAY-OIL AMENDMENT[1]

WILLIAM S. STEWART[2] AND WALTER EBELING[3]

Introduction

Spraying citrus trees with emulsions of oil in water is a standard practice in citrus-pest control. Such sprays are very effective and offer cost advantages over other methods of insect control. Oil sprays, however, may induce leaf and fruit drop (4). Die-back of small twigs, granulation, and water spot (1, 2, 5) are a few of the other indictments against the use of oil sprays.

In general, there is an inverse correlation between the unsulfonated residue (U.R.) of the spray oil (4) and the amount of leaf drop, fruit drop, and die-back of small twigs. The higher the unsulfonated residue, other factors being equal, the fewer these undesirable effects. For this reason the percentage of unsulfonated residue in the oil has become an important specification of spray oils. Oils now used in California are 90–94 U.R., but even at these values leaf and fruit drop often occur to an undesirable extent after a single annual spraying.

Plant growth-regulators are known to be very effective in preventing preharvest fruit drop of certain varieties of apples (8). These substances have also been shown to prevent leaf drop from cut branches of several plant species (9, 11), and their use to decrease fruit drop in oranges has been indicated (6). These facts led to consideration of the use of plant growth-regulators as spray-oil amendments to limit the undesirable effects of oil sprays. The present paper reports preliminary observations on reduction in leaf and fruit drop as the result of adding plant growth-regulators to spray oil used on Washington Navel and Valencia orange trees.

Experimentation

Exploratory tests

Mature leaves on a Washington Navel orange tree were painted with undiluted spray oil with or without 0.1% 2,4-dichlorophenoxyacetic acid (2,4-D). In this as in all subsequent work, the oils used corresponded to the California grade "light-medium" (52–61% distilled at 636° F.). Table 1 lists other properties of these oils. To accentuate leaf drop, 60 (instead of 92) U.R. oil was used. The leaves painted with oil containing 0.1% 2,4-D were more securely attached to the stem after 10 days than were those painted with oil alone. Repeated tests gave similar results.

Next, two branches on the same tree were given a drenching spray with the undiluted oil, with and without 0.1% 2,4-D. Application was made with a 1-quart continuous-pressure garden sprayer. Two weeks after spraying there was a striking difference between the two branches, the one treated with 2,4-D retaining most of its leaves and fruit, in contrast to the other, which was completely defoliated and had lost most of its fruit (fig. 1). This test was repeated twice with the same result.

[1] Paper no. 547, University of California Citrus Experiment Station, Riverside, Calif.

[2] Assistant Plant Physiologist and [3] Associate Entomologist, California Agricultural Experiment Station.

Additional, comparable tests were made, using 2,4-D, as well as phenoxyacetic acid, naphthoxyacetic acid, naphthaleneacetic acid, and naphthalene acetamide, singly and in combination with 2,4-D. In these tests, as in others in which various growth-regulating substances were used to limit preharvest drop in certain varieties of apples (3), 2,4-D alone was found to be the most effective.

The minimum effective concentration of 2,4-D, in limiting leaf drop, in oils of 60, 71, 80, and 92 U.R. was also determined. Concentrations of 0.005, 0.025, 0.05, and 0.1% 2,4-D were prepared in each oil by dissolving 2,4-D in ethyl alcohol and evaporating off the alcohol after addition to the oil. The undiluted oils were sprayed, as described, on single branches. A separate tree was used for each concentration of 2,4-D in each oil—a total of sixteen trees. For comparison, a second, similar branch on each tree

TABLE 1

PHYSICAL PROPERTIES OF THE LIGHT-MEDIUM NAPHTHENIC OILS USED IN PREPARING OIL-WATER SPRAY EMULSIONS

Oil no.	Unsulfonated residue (%)	Temperature (° F.) for distillation of		Saybolt viscosity at 100° F. (secs.)	Gravity (60°/60° F.)	Refractive index (n_D^{20})
		5%	90%			
1	60	570	698	75	0.925	1.516
2	71	443	705	48	.887	1.490
3	80	501	703	55	.883	1.485
4	92	571	695	71	0.886	1.482

FIG. 1.—Branches of Washington Navel orange tree, showing effect on leaf and fruit drop of applying 60 U.R. oil spray (*left*) undiluted and (*right*) with 0.1% 2,4-dichlorophenoxyacetic acid added.

was sprayed with the same oil lacking 2,4-D.

Concentrations of 0.025% 2,4-D, or higher, in all the oils, were very effective in limiting leaf drop. The effects were similar to those shown in figure 1. No differences in leaf drop were noted between control branches (sprayed with oil only) and those treated with 0.005% 2,4-D in oil. The 0.05% treatment was more effective in limiting leaf drop than the 0.025%; there were no apparent differences between the 0.05% and 0.1% 2,4-D treatments. In these trials all concentrations of 2,4-D in oil were equally effective in decreasing fruit drop by 50%, compared with that on branches sprayed with oil alone. It is of interest that the most effective concentration for delaying petiole abscission in *Coleus*, in tests with a large number of growth-regulators, was found to be 0.05% (7).

On the basis of these data, three field trials were made.

FIELD TEST NO. 1

PROCEDURE.—This test was with Washington Navel orange trees in an orchard at Riverside, California, and consisted of a Latin-square arrangement of five treatments. Because of the size and shape of the orchard, a sixth treatment could not be included in the square but was made on adjacent trees. All trees had previously been sprayed with oil, in April, 1945, and the fruit had been picked in December of that year.

Treatments in the square were: (1) no oil spray (control); (2) 92 U.R. oil spray, at 2% concentration in water; (3) same as 2, but with 0.005% 2,4-D in the oil; (4) same as 2, but with 0.025% 2,4-D added; (5) 80 U.R. oil spray, at 2%, containing 0.05% 2,4-D. Treatment 6, outside the square, was the same as 5, except that 2,4-D was omitted. Since the relative solubility of 2,4-D in oil and water is not known, the concentration of 2,4-D is expressed on the basis of its concentration in the oil rather than in the final oil-water emulsion. If 2,4-D were as soluble in water as in oil, its concentration on the emulsion basis would be 0.001% (10 p.p.m.) for the 0.05% 2,4-D in oil used in the 2% oil emulsion.

All spray emulsions were tank-mixed, using 4 ounces of blood albumin per 100 gallons of spray as spreader. The 2,4-D was first dissolved in ethyl alcohol and was then added to the oil as described. The spray was applied in the usual manner on February 18 and 19, 1946, with a power sprayer, using 400 pounds pressure and no. 8 disks. Contamination of control trees by drifting 2,4-D spray was prevented by completely covering them with canvas tents when spraying near by. All leaves were raked from under the trees at the beginning of the experiment.

In this trial, as in all field trials, leaf-drop counts were made by determining the number of leaves within a rectangular frame approximately 1 × 10 feet. This frame was always placed in a southeasterly direction against the tree trunk and extended several feet out beyond the drip of the tree.

RESULTS.—One week after application, trees sprayed with 80 U.R. oil alone (treatment 6) had dropped many more leaves than those sprayed with the same oil containing 0.05% 2,4-D (treatment 5). There was a similar difference between the unsprayed (control) trees and the oil-sprayed ones. At this time leaf-drop differences were not obvious between the trees sprayed with 92 U.R. oil with and without 2,4-D.

To determine whether 2,4-D had induced any differences in leaf drop, five leafy twigs per treatment were cut from

the trees 1 week after spraying and were placed in an atmosphere of ethylene and high humidity in the laboratory. Forty-eight hours later, under these conditions, the total numbers of leaves remaining on each set of five twigs were 0, 1, 5, 10, 18, and 5, respectively, for treatments 1 through 6. These results indicate the effectiveness of treatments 3 and 4 (0.005 and 0.025% 2,4-D in oil) in limiting leaf drop on trees sprayed with 92 U.R. oil, although differences were not yet apparent in the field.

The obvious difference observed in the field between treatments 5 and 6 (80 U.R. oil with and without 0.05% 2,4-D) was also shown in a laboratory ethylene treatment one week after spraying. However, a similar laboratory test made approximately 18 hours after spraying showed no differences between the oil-sprayed leaves with or without 2,4-D. It appears that 2,4-D in oil became effective in limiting leaf drop between 18 and 168 hours after application.

Leaf-drop counts in the field on March 5, over 2 weeks after spraying, showed that addition of 2,4-D to the spray oil resulted in a significant decrease in leaf drop, in comparison with that of trees sprayed with the same oil without 2,4-D (table 2). With 92 U.R. oil containing 0.025% 2,4-D, the decrease was 26.5%; with 80 U.R. oil with 0.05% 2,4-D, the decrease was 57.4%. Even with the addition of 2,4-D, however, the 92 U.R. oil sprays induced about three times as much leaf drop as in unsprayed trees. Leaf-drop counts on March 25 showed comparable differences. Fruit-drop observations on these trees were not possible, as the fruit had been harvested.

Field test no. 2

Procedure.—This test, with Washington Navel orange trees in an orchard at La Verne, California, consisted of a Latin-square arrangement of four treatments: (1) no oil spray (control); (2) 92 U.R. oil spray, at 1.66% concentration; (3) same as 2, but with 0.0037% 2,4-D added to the oil; and (4) same as 2, but with 0.0185% 2,4-D added to the oil.

The required amounts of 2,4-D were added as a commercial liquid concentrate of 40% 2,4-D as the diethanol ammonium salt, instead of the 2,4-D free acid being dissolved in alcohol as in the previous trial.

To avoid effects of spray drift, the treated trees were separated from one another by two trees in each direction. The trees were about 35 years old and had been fumigated but had never before been sprayed with oil. The sprays were applied February 20, 1946, when the fruit on the trees was mature. All leaves and fruit on the ground under the trees were removed.

Although the trees were as uniform as could be expected, there was still variation in size. It was suggested that leaf drop might be correlated with tree size and that a correction for tree volume would be desirable. An analysis of covariance (10) gave a nonsignificant F value, indicating that it made no difference whether leaf drop was corrected for tree volume.

Results.—The numbers of leaves dropped per tree by March 5, 1946, for treatments 1 through 4, were 40, 211, 296, and 162, respectively (table 2). The addition of 0.0185% 2,4-D to oil applied at 1.66% concentration in the emulsion did not significantly limit leaf drop. Counts on March 26 likewise failed to show a statistically significant difference in leaf drop of trees sprayed with oil plus 2,4-D, in comparison with those sprayed with oil alone.

Fruit drop at La Verne for the period

March 21–April 25, from the trees sprayed with 0.0037% 2,4-D oil amendment, was 32.4% less than that from trees sprayed with oil only (table 2). This difference was significant at the 5% level. The decrease in fruit drop for the spray

Fruit-drop counts prior to March 21 showed a trend similar to that reported above, but the differences lacked significance at the 5% level. This may indicate that 2,4-D applied in oil required approximately a month to become effective in

TABLE 2

EFFECT OF OIL SPRAY, WITH AND WITHOUT ADDED 2,4-DICHLOROPHENOXYACETIC ACID, ON LEAF AND FRUIT DROP OF WASHINGTON NAVEL AND VALENCIA ORANGE TREES

TREATMENT NO.	SPRAY OIL		% 2,4-D IN OIL	NO. TREES	NO. LEAVES DROPPED	NO. FRUITS DROPPED
	% U.R.	% in emulsion				
	Field test no. 1 (Washington Navels, Riverside)*					
1 (control)				5	94	
2	92	2.00	0.0000	5	411	
3	92	2.00	.0050	5	397	
4	92	2.00	.0250	5	302	
5	80	2.00	.0500	5	571†	
6	80	2.00	0.0000	5	1341	
	Field test no. 2 (Washington Navels, La Verne)‡					
1 (control)				4	40	120.2
2	92	1.66	0.0000	4	211	179.0
3	92	1.66	.0037	4	296	120.7
4	92	1.66	0.0185	4	162	123.6
	Field test no. 3 (Valencias, Riverside)					
1 (control)				8	74	
2	92	2.00	0.0000	8	402	
3	92	2.00	.0050	4	369	
4	92	2.00	.0250	4	283§	
5	80	1.50	.0000	2	521	
6	80	1.50	0.0500	2	400	

* Least significant difference at 5% level, 102 leaves.
† Compared with same spray without 2,4-D, difference significant at 1% level. (Treatment not in Latin square.)
‡ Least significant difference at 5% level: leaves, 132; fruits, 58.
§ Compared with same spray without 2,4-D, difference significant at 5% level. (Treatment not in Latin square.)

containing 0.0185% 2,4-D was 30.9%. The unsprayed trees dropped 32.8% less fruit than those sprayed with oil alone. Fruit loss induced by the oil spray was thus counteracted by the addition of 2,4-D.

limiting fruit drop. GARDNER (6), for example, found that naphthaleneacetic acid and naphthalene acetamide were most effective in decreasing drop of Pineapple oranges in Florida when applied several months before harvest.

A concentration of 0.0037% 2,4-D in oil was effective in limiting fruit drop, whereas the lowest effective concentration for a significant decrease in leaf drop seems to be between 0.0185% and 0.025%. This is a concentration at least five times greater than that required for a significant decrease in fruit drop. This result is in agreement with the observation by GARDNER and COOPER (7) that "the prevention of leaf abscission appears to require a much higher concentration of applied growth substance than the prevention of mature fruit drop."

FIELD TEST NO. 3

PROCEDURE.—This test was with Valencia orange trees in an orchard at Riverside, California. The trees were sprayed on February 22, 1946. The six treatments were: (1) no oil spray (control); (2) 92 U.R. oil spray, at 2% concentration in water; (3) same as 2, but with 0.005% 2,4-D added to the oil; (4) same as 2, but with 0.025% 2,4-D added; (5) 80 U.R. oil spray, at 1.5% concentration in water; (6) same as 5, but with 0.05% 2,4-D added to the oil.

There was at least one untreated tree (usually there were two) between the trees in treatments 1, 2, and 5 (no 2,4-D used) and the trees in treatments 3, 4, and 6 (sprayed with 2,4-D in oil). Except for this restriction, the treatments were randomized among trees in two rows. The numbers of trees used for treatments 1 through 6 were: 8, 8, 4, 4, 2, and 2, respectively. Maturing fruit was on the trees. Oil emulsions and 2,4-D additions were prepared and applied as in the La Verne trials.

RESULTS.—Counts on March 9 indicated a 29.6% decrease in leaf drop (significant at the 1% level) owing to the addition of 0.025% 2,4-D to the 92 U.R. oil (table 2). With the 80 U.R. oil, the decrease in leaf drop resulting from the addition of 0.05% 2,4-D was 23.2%. Since only two trees were available for each treatment with the 80 U.R. oil, the significance of this decrease could not be statistically tested.

Leaf-drop determinations on March 25 showed differences similar to those above. There was no obvious drop of the immature fruit as a result of the oil spray. It will be of interest to note whether 2,4-D oil spray applied to trees with immature fruits will decrease the drop of the mature fruits.

The above data indicate that the positive results in the second and third field tests are due to 2,4-D and not to the diethanolamine in which it was added, since they are the same as in the previous trials in which no diethanolamine was used.

TREE INJURY

The possibility of more tree damage from 2,4-D than from the oil spray is a consideration of prime importance. It has been generally observed that 2,4-D in water solutions of 0.1% has a deforming effect on expanding leaves and stems of the orange tree. In July, 1945, two 10-year-old Valencia orange trees with no expanding leaves or buds were sprayed with 2,4-D (approximately 0.14%) in water. Until November of that year, no effects of the spray were apparent, except for a lack of new growth; owing to the location of the trees, it was difficult to determine whether this was the result of 2,4-D injury. Several trees sprayed at the same time with 0.07% 2,4-D in water also failed to show new growth. Valencia trees sprayed September 15, 1945, with 0.14% 2,4-D in water showed epinastic curvatures of the actively growing stems and leaves and a yellowing of the expanding leaves. The plant parts that were not

expanding made no externally visible response. In all cases observation of the roots disclosed no indications of injury. Since all these trees were destroyed when the tract was cleared, in November, 1945, additional observations were not possible. On other trees left standing in the same grove, on which only isolated actively growing branches had been sprayed in September, 1945, the effects described above were still persistent in April, 1946. No lateral buds were then expanding on the sprayed branches.

A 15-year-old grapefruit tree sprayed September 15, 1945, with 0.14% 2,4-D in water, responded in the same manner as the oranges. The shoots that were actively growing at the time of spraying were still distorted in April, 1946, and showed no tendency to become normal. This response and the apparently delayed opening of the flower buds were the only obvious effects of 2,4-D. The roots showed no changes because of the treatments and appeared to be healthy.

A 5-year-old lemon tree with many actively growing shoots was sprayed at the same time (September 15) and in the same manner as the grapefruit. Severe epinastic curvatures of the growing stems and leaves occurred within 10 days. After one month, the pedicels on sprayed trees were thickened in comparison with those on nonsprayed trees, and the fruit appeared to be very securely attached. By December many apparently mature leaves had become a dull-yellow color.

As expected from the above observations, the stage of bud expansion appears to be an important factor in the use of 2,4-D as a spray-oil amendment. At the time of spraying the Riverside Navel plot (field test no. 1) many stem and flower buds had expanded to a length of 2 mm., and young leaves were 3–7 mm. long. Three weeks after spraying with 0.025% 2,4-D, the lateral halves of the basal portions of the leaf blades were expanding normally but the apical halves remained closed. By April 15, however, most blades had opened normally their entire length, and none remained tightly closed. The expanding leaves on the trees sprayed with 0.05% 2,4-D curled tightly inward and had failed to open in the 2 months after spraying. By July, when the next growth flush occurred, these leaves were expanded fully, except for a slight pinching-together of the blade near the tip. No effect on leaf growth was observed from the 0.005% 2,4-D spray. Opening of flower buds was apparently normal in all treatments.

In contrast to the Riverside Navels, the Riverside Valencias and the La Verne Navels were in a tighter bud stage at the time of spraying and, in general, the expanding vegetative and flower buds showed no undesirable responses, even to the 0.05% 2,4-D spray. Only rarely were the above-described effects observed on these trees. Possibly the few scattered buds showing a response were further expanded than the others at the time of spraying.

To avoid the deforming effects resulting from sprays containing 2,4-D, application should be made only when the tree has a minimum of actively growing branches and not during a growth flush. In general, this corresponds with the times when sprays are usually applied. These observations all forcibly emphasize the fact that the use of 2,4-D as a spray-oil amendment must await further study of undesirable effects on the trees. Such studies are now in progress.

Discussion

The addition of 0.025% 2,4-D to 92 U.R. tank-mixed oil used at 2% concentration in water on Washington Navel and Valencia orange trees at Riverside (table 2) significantly limited leaf drop,

in comparison with results from the same oil spray used without 2,4-D. The use of a similar spray oil at 1.66% concentration, with 0.0185% 2,4-D, on Washington Navels at La Verne, also decreased leaf drop, but the difference between these results and those obtained with the same oil spray without 2,4-D was not statistically significant. The difference in the results at Riverside and La Verne was apparently not caused solely by the lower concentration of 2,4-D used at La Verne but was partly the result of the smaller oil deposit on the leaves in the La Verne test, owing to the lower percentage of oil in the emulsion; this was indicated by direct determinations of the amount of spray oil deposited on the leaves.

Duplicate analyses for deposited oil were made on 100 leaves per treatment, collected equally from all trees in the treatment on the day following spraying. For treatments 2 (92 U.R. oil only, at 2%) and 4 (the oil plus 0.0185% 2,4-D), the amounts of deposited oil on the Navel orange leaves from La Verne were, respectively, 65.8 and 53.3 ml. $\times 10^{-6}$/sq. cm. of leaf area. For the corresponding treatments on the Valencia leaves from Riverside, the amounts of deposited oil were, respectively, 69.1 and 83.6 ml. $\times 10^{-6}$/sq. cm. The Valencia trees showed a significant decrease in leaf drop, even though there was actually more oil deposited on the leaves of the trees sprayed with oil plus 2,4-D than on those sprayed with oil alone. In the La Verne trials the comparison failed to show a significant decrease in leaf drop, even though in this case less oil was deposited on the leaves treated with 2,4-D than on those with oil alone.

Assuming that all the 2,4-D added was carried in the oil in the emulsion, the maximum amounts of 2,4-D per square centimeter of leaf surface of the Riverside Valencias and La Verne Navels may be calculated to have been 0.0209 μ gm. and 0.0099 μ gm., respectively. The amount of 2,4-D actually effective in the La Verne trial was 53% less than in the Riverside trial, instead of 26% less, as it would have been on the basis of 2,4-D concentration alone. If the upper and lower leaf surfaces are assumed to total 50 sq. cm., the amount of 2,4-D deposited per leaf on the Valencia trees was 1.04 μ gm. Since this amount is probably very small compared with that of the exploratory trials, where branches were sprayed with undiluted oil with 2,4-D added, tests are being made with oil-emulsion sprays containing higher concentrations of 2,4-D than those reported here.

Although the foregoing comparison is between two varieties of oranges (Washington Navel and Valencia), the observed differences in leaf drop because of varietal differences are not considered significant here, since at Riverside both varieties gave the same response to the same treatment.

Summary

1. Exploratory tests indicated that the addition of 0.025% 2,4-dichlorophenoxyacetic acid to undiluted spray oil applied to branches of both Washington Navel and Valencia orange trees strikingly limited leaf and fruit drop, in comparison with that of trees similarly sprayed, but without 2,4-D.

2. In preliminary field trials the addition of 0.025–0.05% 2,4-D to the oil in oil-water emulsion sprays for use on orange trees significantly decreased leaf drop 26.5% to 57.4%, the percentage reduction depending on the properties of the oil and on the concentration of 2,4-D.

3. In the one trial where fruit counts were possible, the addition of 0.0037% 2,4-D to the spray oil significantly de-

creased fruit drop 30.9%, compared with drop from trees similarly sprayed, but without 2,4-D.

4. Experiments have shown that the stage of bud expansion appears to be an important factor in the use of 2,4-D as a spray-oil amendment. At the present time, 2,4-D as a spray-oil amendment cannot be recommended for commercial orchard practice, since under certain conditions not yet fully established injury to trees may result from its use.

The authors thank the California Spray Chemical Company of Pittsburg, California, and the Thompson Horticultural Chemicals Corporation of Los Angeles, respectively, for supplying spray oils and plant growth-regulators. They appreciate, also, the generous co-operation of Mr. M. A. McDERMONT of Riverside and Mr. E. GRAHAM of the Peyton Corporation, La Verne, in making trees available for these studies.

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UNIVERSITY OF CALIFORNIA
CITRUS EXPERIMENT STATION
RIVERSIDE, CALIFORNIA

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ANALYSIS OF LEVULINS, INULIN, AND MONOSACCHARIDES IN GUAYULE[1]

HAMILTON P. TRAUB[2] AND M. C. SLATTERY[3]

Introduction

In previous papers the nature of the water-soluble carbohydrates in guayule (*Parthenium argentatum* A. Gray) has been established (**2, 5, 11, 12**), and the importance of relating the relative proportions of the 89%-ethanol-soluble and the 89%-ethanol-insoluble levulins to plant functioning has also been indicated (**11**). The reader is referred to TRAUB and SLATTERY (**11**) for criticism of the earlier untenable conception of the nature of the chief carbohydrate reserve in guayule (**4**). In the present paper methods of extracting and analyzing for water-soluble carbohydrates and for distinguishing between 89%-ethanol-soluble and 89%-ethanol-insoluble levulins are briefly summarized. Typical results obtained by the application of these methods, showing seasonal changes in guayule tissues, are presented.

Methods

EXTRACTION OF TISSUES

The tissue samples are dried, finely ground, and then again dried at 65° C., according to the procedure described by TRAUB (**9**). The method for the extraction of water-soluble carbohydrates is adapted from the work of TRAUB *et al.* (**13**) and consists of (*a*) cold-water extractions for the determination of total free sugars, total free fructose, and total cold-water-soluble carbohydrates (free sugars plus levulins) and of (*b*) hot-water extractions for the determination of total water-soluble carbohydrates (free sugars, levulins, and inulin).

COLD-WATER EXTRACTION.—Duplicate 2-gm. samples of the finely ground tissue, prepared as described by TRAUB (**11**), are transferred to 100-ml. volumetric flasks, provided with glass stoppers, by means of a stemless glass funnel. Approximately 1 gm. of powdered $CaCO_3$ and 50 ml. of water are then added, and the tissue is wetted by rotating the flasks, which are then placed in a water bath at 20° C. for 1 hour. After removal, 1 ml. of a saturated solution of neutral lead acetate is added, and the contents of the flasks are made up to volume and thoroughly mixed. After standing for a few minutes to allow for complete precipitation of proteins, tannins, etc., the contents are filtered into flasks with 0.2–0.3 gm. of dry powdered sodium oxalate on the bottom. After shaking, the contents of the flasks are allowed to stand for a few minutes to insure complete deleading and are then filtered into 125-ml. dry Erlenmeyer flasks. Aliquots of the filtrate are used for the determination of total free sugars, total free fructose, and total cold-water-soluble carbohydrates (free sugars plus levulins).

HOT-WATER EXTRACTION.—The pro-

[1] Contribution from the Emergency Guayule Research Project, Bureau of Plant Industry, Soils and Agricultural Engineering, Agricultural Research Administration, U.S. Department of Agriculture. The co-operation of the U.S. Forest Service is gratefully acknowledged.

[2] Principal Physiologist, Bureau of Plant Industry, Soils and Agricultural Engineering, U.S. Plant Industry Station, Beltsville, Md.

[3] Formerly Assistant Physiologist, Bureau of Plant Industry, Soils and Agricultural Engineering.

cedure is the same as that outlined in the preceding paragraph except that the flasks containing the wetted tissue are placed in a boiling-water bath for 1 hour, and the neutral lead acetate is added after cooling the contents of the flasks to 20° C. in a water bath. Aliquots of the final filtrate from these extractions are used for the determination of total water-soluble carbohydrates (free sugars, levulins, and inulin).

At times considerable difficulty may be encountered in filtering hot-water extracts of guayule leaves and new growth, apparently because of the colloidal state of some of the constituents extracted with hot water. In such cases, one or two spoonfuls of diatomaceous earth are placed on the filter paper before the plant extracts are poured on.

Carbohydrate analysis

Aliquots from the cold-water extractions, without hydrolysis, are used for the determination of total free sugars by Somogyi's semimicro copper method (**8**) as modified by Browne and Zerban (**1**) and of total free fructose by Nyn's cupro-carbonate method (**6**) as modified by Jackson and Mathews (**3**).

Levulins, or levulins plus inulin, in 10-ml. aliquots from cold- or hot-water extractions, are hydrolyzed with 1 ml. of 10% HCl (1.0741 sp. gr.) in a hot-water bath at 70° C. for 10 minutes. The solutions are neutralized with dilute NaOH, with phenolphthalein as indicator. Aliquots are then taken for the determination of total cold-water-soluble carbohydrates (free sugars and inulin) and total water-soluble carbohydrates (free sugars, levulins, and inulin), respectively, again by Somogyi's semimicro copper method (**8**) as modified by Browne and Zerban (**1**).

For the rapid determination of total fructose (free and polymerized) in the cold- or hot-water extracts, Roe's alcoholic resorcinol method (**7**), or this method as modified by McRary and Slattery (**4**), may be used. The following values are determined directly: (*a*) total free sugars (from cold-water extract); (*b*) total free fructose (from cold-water extract); (*c*) free sugars plus levulins (from cold-water extract); (*d*) free sugars, levulins, and inulin (from hot-water extract); and (*e*) total fructose, free and polymerized (from both cold- and hot-water extracts, respectively). The required values not directly determined are obtained by subtraction: nonfructose free sugars, $a - b$; levulins, $c - a$; and inulin, $d - c$.

Fractionation of levulins

The basic values for free sugars plus 89%-ethanol-soluble levulins are determined as follows: 1- to 2-gm. samples of the finely ground tissue are continuously extracted with 89% ethanol, by volume, in a reflux apparatus, Landsiedl type, for 6 hours. However, the Soxhlet type extractor may be substituted if desired. For purposes of extraction, 80% ethanol, by volume, is placed in the boiling flask of the extractor, and thus the condensate dripping into the extraction cups will be approximately 89% ethanol by volume (**11**). The insoluble residue is discarded. The flask containing the ethanol extract is placed on the steam bath, and the ethanol is evaporated off. The 89%-ethanol-extract residue is transferred quantitatively to 100-ml. flasks and is made up to volume with water. Aliquots of this solution, hydrolyzed with HCl, neutralized with dilute NaOH, and clarified as indicated in the previous section, are used for the determination of free sugars plus 89%-ethanol-soluble levulins

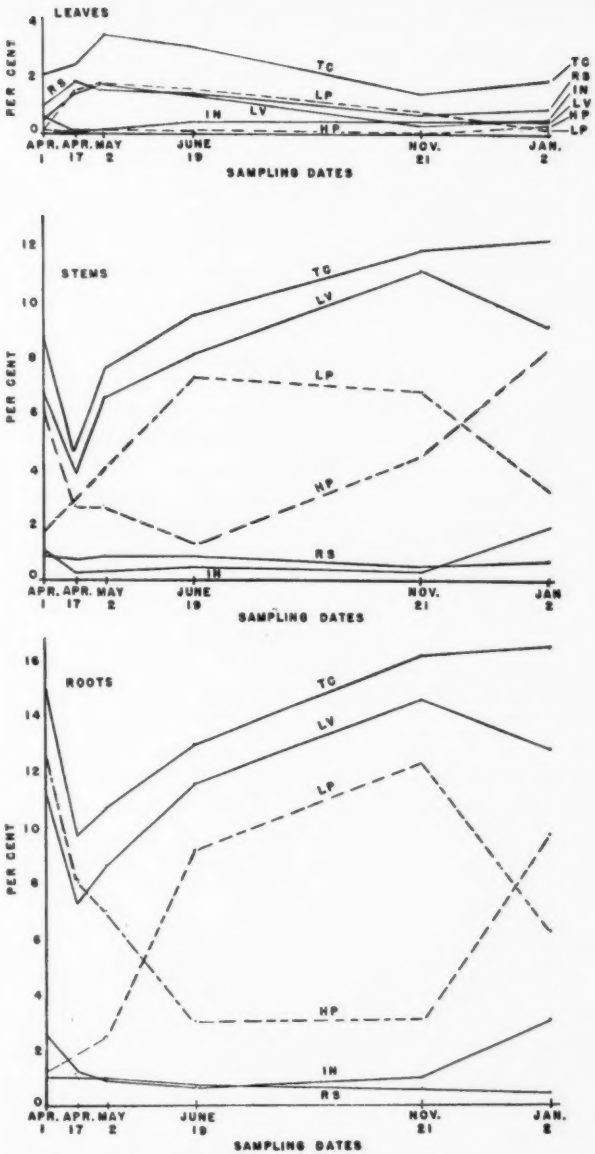

FIG. 1.—Seasonal changes in 2-year-old guayule nursery plants—leaves, stems, and roots; free sugars (*RS*); levulins (*LV*); inulin (*IN*); 89%-ethanol-soluble levulins (*LP*); 89%-ethanol-insoluble levulins (*HP*); and total water-soluble carbohydrates (*TC*); expressed on dry-weight basis.

by SOMOGYI's semimicro copper method (8) as modified by BROWNE and ZERBAN (1).

The values for the 89%-ethanol-soluble levulins are obtained by subtracting the values for free sugars from the values for free sugars plus 89%-ethanol-soluble levulins. The values for the 89%-ethanol-insoluble levulins are obtained by subtracting the values for free sugars plus 89%-ethanol-soluble levulins from the values for total water-soluble carbohydrates (free sugars, total levulins, and inulin).

Seasonal changes

Typical results obtained by the application of the methods briefly described above are shown graphically in figure 1. Seasonal changes in the various fractions of the water-soluble carbohydrates in guayule leaves, stems, and roots will be briefly discussed.

Free sugars (*RS*) are relatively limited in amount in the stems and roots, below 1%, but rise to almost 2% in the leaves. As contrasted with the relatively greater variation in levulins and inulin, there is little seasonal variation in percentage of free sugars in stems and roots, suggesting that the latter are the carbohydrates of translocation in guayule.

The inulin fraction (*IN*) is relatively small, never rising as high as 4%, in this case, in any of the tissues; it is largest in the roots, somewhat smaller in the stems, and very limited in the leaves. The inulin values rise with decrease in temperature in November, reach a peak in midwinter, and in April begin to fall again to the summer low.

Levulins (*LV*) are the chief reserve carbohydrates in the stems and roots of guayule at all seasons. The percentage of levulins is always highest in the roots, in which it falls to a minimum value soon after the first flush of growth in April. It rises rapidly until June and then gradually to the midwinter peak.

When the relative proportion of the 89%-ethanol-soluble levulins to the 89%-ethanol-insoluble levulins (11) is considered, we obtain a more dynamic picture of the carbohydrate transformations in guayule tissues. The values for 89%-ethanol-soluble levulins (*LP*) rise to a high during the summer and fall to a low in winter, beginning with the onset of lower temperatures, whereas the values for 89%-ethanol-insoluble levulins (*HP*) present a mirror image, falling to a low in summer and rising to a high in winter. TRAUB *et al.* (10) have shown that drought, or high soil-moisture, stresses, applied to guayule nursery seedlings, have an effect similar to that of low temperature.

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CURRENT LITERATURE

Die Koniferen des Oberkarbons und des unteren Perms. By RUDOLF FLORIN. *Palaeontographica* **85**, Abt. B (Hefte 1–7): 1–654. 1938–1944. Stuttgart. With pls. 1–186.

For some time, with interruption caused by World War II, Dr. RUDOLF FLORIN has been publishing in *Palaeontographica* his series of researches on Paleozoic conifers. These have appeared: Part 1 in 1938, Parts 2–4 in 1939, and Part 5 in 1940. Nearly all these earlier issues have reached American libraries. The printing of Parts 6–8 was delayed; Parts 6 and 7 bear 1944 dates and have thus far appeared in very few American libraries, while Part 8, which includes the summary, the all-important bibliography for Parts 6–8, and the index for the entire series, is in type but still awaits printing.

FLORIN has carefully examined at first hand fossil materials of the Upper Carboniferous, Permian, and Mesozoic. He gives detailed descriptions of hundreds of items found in European and American collections, all fully illustrated in 150 quarto plates (Parts 1–4). Here FLORIN applies his detailed knowledge of stomatal and cuticular anatomy founded upon his well-known researches[1] on these structures in the living conifers.

The fossil material of Paleozoic conifers is very abundant, but nearly all of it is in the form of impressions and compressions. Only the latter include some organic residues. True petrifactions are not available. Specimens of petrified Paleozoic wood are only doubtfully coniferous. In spite of the unsatisfactory condition of these fossils, it was possible to bring together the specimens belonging to many species under various genera through comparison of their cuticular characters and to recognize the reproductive parts that belong to the same system.

FLORIN has segregated from the fossil material, which had been referred to *Walchia* Sternb. more than a century ago, two Upper Carboniferous and Lower Permian genera: *Lebachia* Florin (Parts 1–3), under which he recognizes fourteen species, and *Ernestiodendron* Florin (Part 4), for which he describes several species. Others are left, and several new species are placed, under *Walchia* and related either to *Lebachia* or *Ernestiodendron*.

Part 5 includes about fifteen form genera, many of them based upon reproductive parts. These have all been subjected to the same detailed treatment and are illustrated in plates 151–166. Also included in Part 5 are keys to these fossil genera and a bibliography of 282 titles specifically for Parts 1–5.

Parts 6 and 7, dealing in summary with the vegetative and reproductive organs of fossil and modern conifers, are most interesting to the reviewer. These chapters include more general discussions, reconstructions, and comparisons. In addition to plates 167–186, there are 65 text figures, many of them in full page, showing FLORIN's restorations of the Paleozoic and Mesozoic conifers.

The Lebachias were monoecious plants of moderate size, having small leaves always spirally arranged, small cones, and small seeds. Their growth habit resembled that of *Araucaria excelsa*. The leaves were small, closely imbricated, awl-shaped, slightly dimorphic, and with a single mid-vein. Those clothing the main stem and leaders were slightly larger than those on smaller secondary branches and had the apex forked, as in leaves of *Gomphostrobus*. Smaller leaves had acute, unforked apices.

The seed cones and pollen cones of *Lebachia* were

[1] R. FLORIN, Untersuchungen zur Stammesgeschichte der Coniferales und Cordaitales. I. Morphologie und Epidermisstruktur der Assimilationsorgane bei den rezenten Koniferen. K. Svenska Vet.-Akad. Handl. 3. ser. Vol. **10**. Stockholm. 1931.

terminal on short lateral branches and always monosporangiate. No evidence was uncovered to indicate bisporangiate cones. The pollen cones were simple strobili with narrowly triangular, peltate, acute sporophylls spirally imbricated on the cone axis. Two microsporangia were attached beneath the sporophyll shield on opposite sides of the stalk. The pollen grains were peculiarly winged all around, with the exoexine almost wholly surrounding the included cell.

The seed cones were compound structures having the reproductive parts borne on short shoots or fascicled in the axils of bracts. The subtending bracts were forked at the tips after the pattern of the *Gomphostrobus* leaf type. In the axils of these bracts occurred free sterile and fertile stalks (interpreted as telomes by the author) intermingled. The seeds were carried singly and erect at the tips of the fertile appendages. *Ernestiodendron* was very similar to *Lebachia*, but some of the other genera of the period (various *Walchia* species) differed in having the seeds inverted at the ends of the stalks.

Part 7 includes descriptions of the reproductive parts of Cordaitales of two principal types. This is followed by a chapter of comparison of the Paleozoic conifers with the Cordaitales. Also discussed are the Upper Permian conifers, *Pseudovoltzia* Florin and *Ullmannia* Goeppert. As FLORIN reconstructs the cone-scale complex of *Pseudovoltzia*, it is composed of nine members unfused, except possibly at the base: three inverted ovules are borne at the ends of as many long stalks, behind which are five sterile leaflike members, and the lanceolate bract which subtends this assembly. It is through fusion of these members with each other and finally with the bract that the cone scale of the Pinaceae, Araucariaceae, Taxodiaceae, and other conifers has been derived.

FLORIN also considers a selection of generic types from the Mesozoic, with his own diagrammatic reconstructions and interpretations. This discussion considers about twenty Mesozoic conifers, with the Taxales set up as a separate order. The living conifers (with fifty genera) are then interpreted in the light of the fossil evidence presented. They are described under Taxodiaceae, Pinaceae, Araucariaceae, Podocarpaceae, Cupressaceae, and Cephalotaxaceae. The four genera of Taxaceae—*Taxus*, *Austrotaxus*, *Torreya*, and *Amentotaxus*—are here included under the Taxales, as an order distinct from the Coniferales. He finds *Callitropsis* Compton (not Oersted) a later homonym and supplies the new name *Neocallitropsis*.

There can be no question about the immense value of the study of cuticular anatomy to the identity of the conifers, other gymnosperms, and pteridophytes. Through this approach, FLORIN has been able to recognize and define fossil genera, including the least-known conifers of the Paleozoic. These new monographic studies will be indispensable in a study of conifers from all geological horizons. His cuticular investigations must be considered in any future monographic treatment, including that of living conifers.

From FLORIN's evidence, it appears that the old controversy concerning the nature of the coniferous cone scale has been definitely settled on the basis of historical data. The ovuliferous scale is an axillary structure or brachyblast, entirely separate from the bract in the compound seed cone of these fossils. The ovules, however, are megasporangia borne on their own stalks, not on the surface of the ovuliferous scale. In the course of evolution, these stalks fused with one another, with associated sterile stalks, and eventually with the subtending bract. To speak of the ovuliferous scale as a ligular outgrowth or appendage of the bract or as a "carpel" is obviously erroneous.

The reviewer is not fully convinced that the separation of the Taxales in a separate order is necessary. True, these are the "conifers without cones," meaning seed cones, but they all possess pollen cones and are a part of the conifers. This emphasizes the fact that the Taxales go far back in their history to the early Mesozoic. FLORIN considers that in the Taxales the female reproductive organs are not reduced from complex cones to single ovules but rather that this group never possessed seed cones and that the erect ovule was held over or derived somewhat directly from the erect ovules and seeds found in the Lebachias and earliest conifers. This condition held over, and it is still to be met with in *Voltziopsis* of the Mesozoic.

FLORIN's work in its entirety is requisite for any study of fossil plants. It is equally indispensable as a background to workers in the field of living conifers. —J. T. BUCHHOLZ.